MECHANISMS OF FETAL ALCOHOL SPECTRUM DISORDERS

A Dissertation

by

SHANNON ELIZABETH WILSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Timothy Cudd
Committee Members,	Guoyao Wu
	Randolph Stewart
	Wei-Jung Chen
Head of Department,	Glen Laine

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ABSTRACT

Mechanisms of Fetal Alcohol Spectrum Disorders. (August 2010)

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Chair of Advisory Committee: Dr. Timothy Cudd

Alcohol consumption during pregnancy can result in fetal alcohol spectrum disorders (FASD), which encompass a range of physical, behavioral, learning, emotional and social disturbances. Many mechanisms for this array of alcohol-derived fetal injuries have been proposed, but none fully accounts for the deficiencies observed. Alcohol is a ubiquitous drug that may affect the brain at any or all stages of development and at multiple sites; regional differences in vulnerability of different brain structures during different periods of exposure have been demonstrated.

This study investigates possible mechanisms for the alcohol induced neurodevelopment damage seen as a result of prenatal alcohol exposure, and also includes evaluation of a potential intervention strategy (glutamine). These experiments all utilized the sheep model, which has distinct advantages over the rodent model for third trimester-equivalent studies (a time of increased vulnerability to the effects of alcohol).

The fetal hippocampal formation (pyramidal cells in the CA1 and CA2/3 fields and granule cells of the dentate gyrus) and olfactory bulb (mitral cells) have been altered in response to alcohol exposure in rodent model studies. This study examined the

effects on the fetal hippocampal formation and olfactory bulb in response to all three trimester-equivalent alcohol exposure in the sheep model, a species in which the third trimester-equivalent occurs *in utero* (as opposed to post-natal as occurs in the rodent). It is known that both maternal and fetal cortisol levels increase in response to alcohol. The role of cortisol in mediating fetal cerebellar Purkinje cell loss (known to occur with alcohol exposure) was analyzed. Lastly, the availability of circulating amino acids, both maternal and fetal, in response to alcohol are reported. The results of administration of a single acute dose of glutamine to the ewe, concurrent with alcohol, was evaluated for its ability to prevent the amino acid and pH perturbations known to occur in response to alcohol.

DEDICATION

For Garret and Ty

To God be any and all glory

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1. INTRODUCTION

1.1 Prenatal Alcohol Exposure and the Range of Damage

The first scientific report linking maternal alcohol use in pregnancy with harm to the unborn baby was published in 1968 (Lemoine et al., 1968). The term Fetal Alcohol Syndrome (FAS) was coined and formally identified in 1973 (Jones et. al., 1973). The cardinal features of FAS are facial dysmorphology, pre and postnatal growth retardation, and central nervous system dysfunction (Jones et al., 1973; Sokol and Clarren, 1989). Alcohol abuse during pregnancy can cause a wide range of neurodevelopmental and behavioral deficits, and children exhibiting less severe signs of damage than seen with FAS have been identified as having Alcohol Related Nuerodevelopmental Disorder or ARND (Stratton et al., 1996) or Alcohol Related Birth Defects, also called ARBDs (Sokol and Clarren, 1989). The more inclusive classification of Fetal Alcohol Spectrum Disorders (FASD) is now used to describe the wide spectrum of neurodevelopmental defects that can be manifested in response to prenatal alcohol exposure (Riley and McGee, 2005; Sokol et al., 2003). FASD is an umbrella term that includes the classifications of ARBD and ARND as well as FAS, which remains the most severe manifestation.

Since the identification of the risks associated with drinking alcohol during pregnancy, considerable efforts have been applied to educate women about the dangers of drinking during pregnancy. In spite of these widespread educational efforts,

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This dissertation follows the style of Alcoholism: Clinical and Experimental Research.

the incidence of FAS has not diminished and alcohol is now identified as the leading human teratogen (Caetano, et al., 2006). Prenatal alcohol exposure is the leading preventable cause of retardation in the United States (Abel and Sokol, 1991). FAS (the most severe form of FASD) is estimated to affect 0.5-2.0 per 1000 live births, but when including broader ranges of damage from prenatal alcohol exposure, FASD, the estimate is as high as 10 per 1000 live births (May and Gossage, 2001). The estimated cost of FASD in the United States for the approximately 40,000 children per year born with FASD is $6 billion (Lupton et al., 2004).

1.2 Need for FASD Research

Since the magnitude of this problem is so great and educational efforts have failed to reduce the incidence, it is necessary to explore the development of intervention/amelioration strategies or therapeutics. In order to formulate these strategies, the specific mechanisms by which alcohol mediates neurodevelopmental damage and growth retardation must be identified.

Though few human FAS autopsies exist (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et al., 1983; Coulter et al., 1993), and only a limited number of children with FAS have been examined with non-invasive techniques such as MRI (Mattson et al., 1992, 1994; Sowell et al., 1996, 2001; Archibald et al. 2001, Riley et al., 2004), these cases have identified a number of brain regions that suffer damage as a consequence of fetal alcohol exposure. Affected regions include the cerebellum, hippocampus, neocortex, olfactory bulbs, thalamus, basal ganglia, and the corpus callosum. Animal studies have demonstrated that heavy alcohol exposure during development can produce

deleterious effects in most of the same brain regions as reported in humans with FAS. In addition, animal studies have identified deficits in neuronal numbers in specific brain regions and alterations in the morphology of neurons that survive the alcohol insult (Barnes and Walker, 1981; Hammer and Scheibel, 1981; Hamre and West, 1993; Miller, 1986; Smith et al., 1986: Volk et al., 1981; West et al., 2001).

Human studies are severely handicapped in addressing some of the key issues related to FAS due to variable consumption patterns of alcohol, unreliable self-estimates of alcohol intake by pregnant women, and because many women who abuse alcohol also abuse other drugs (Rosett and Weiner, 1983). Animal model systems allow control over important maternal and environmental variables such as genetic background, nutritional status of the mother, dose and timing of the alcohol exposure as well as other factors that may influence fetal outcome with a degree of control not otherwise possible (Becker et al., 1994; Coles, 1994). Importantly, animal model systems also allow questions to be addressed at a more mechanistic level than would otherwise be possible.

1.3 Sheep Model

Sheep possess several major advantages for the study of FAS when compared with other animal models. The adult ewe and fetus are tolerant of handling, surgery and chronic instrumentation, which facilitates long-term treatment and blood sampling. Surgically placed indwelling vascular catheters can be maintained with fewer complications for a more extended period of time in adult sheep than chronically implanted catheters in the primate. A large literature exists on the normal physiology of fetal sheep, which precludes the need for exhaustive studies just to determine normal

values and to establish the animal preparation. The large body mass of the sheep is also an advantage. The sheep fetus will weigh between 0.85-4.50 kg during the third trimester equivalent (this weight range represents the rapid growth during this period of gestation), while the adults will weigh between 50 and 75 kg, comparable with that of a human fetus and an adult woman respectively. When the measurement of multiple dependent variables must be performed simultaneously and at multiple time points, thus requiring the collection of relatively large volumes of blood, an animal of significant body mass is required. Such experiments cannot be performed in small laboratory animals. The longer length of gestation in sheep allows the investigator more opportunity to intervene or perform experiments at more specific times during gestation, while the short gestation periods of other mammals used for fetal alcohol research (e.g. 22 days for the rat) increase the difficulty in determining critical periods that cause damage. All three trimester-equivalents of pregnancy occur *in utero* in the sheep. The peak velocity of brain growth, called the "brain growth spurt" (Dobbing and Sands, 1973, 1979) is a period of accelerated brain growth thought to be one of enhanced vulnerability to a variety of teratogens. The “brain growth spurt” occurs *in utero* in the sheep similar to humans in which the peak velocity of brain growth is reached at the time of birth. In rats, the third trimester-equivalent and “brain growth spurt” occur postnatally (Dobbing and Sands, 1973; Dobbing and Sands, 1979), so response to alcohol administration during the third trimester-equivalent of human brain development in rats must be conducted postnatally. Therefore, to extrapolate the findings from rats to humans, one must assume that the intrauterine environment, placenta,

mother and parturition play a limited role in mediating the damage (Cudd, 2005).

1.4 Maternal Alcohol Abuse Patterns

Peak blood alcohol concentration (BAC) has been shown to be a good predictor of fetal brain damage in response to prenatal alcohol exposure (Bonthius and West, 1990). Binge drinking (compressing total alcohol consumption into a short period of time with a short time between drinks) results in higher BACs and thus can be more harmful to the fetus (Maier and West, 2001a). It is well known that chronic alcoholics can have BACs well over 200 mg/dl and still appear sober (Maier and West, 2001a; Urso et al., 1981). A binge drinking pattern is common in women who consume alcohol during pregnancy (Caetano et al., 2006; Maier and West, 2001a) and this pattern can be mimicked by employing a three-day binge exposure followed by four days without alcohol (comparable to weekend binging).

2. GAPS IN KNOWLEDGE

In spite of the vast clinical and experimental literature on FASD, the mechanism of action by which alcohol mediates neurodevelopmental damage has yet to be determined with any specificity. Alcohol is a ubiquitous drug that may affect the brain at any or all stages of development (West, 1987). Several possible mechanisms of damage are possible: direct effect on neuronal precursors (Sulik et al., 1984) or dividing or migrating cells (Miller, 1986; Miller and Nowaski, 1991; Miller and Robertson, 1993), disruptive effect on neurons that have been generated and are in different stages of differentiation (Bauer-Moffett and Altman, 1977; Bonthius and West, 1991a, 1991b; Hammer and Scheibel, 1981), or glial development (Goodlett, et al., 1993; Miller and Robertson, 1993; Shetty et al., 1994). Indirect mechanisms of damage include alteration of maternal functions, placental transport, or any key biochemical step involved with fetal development (Becker et al., 1994; West et al., 1994).

This wide spectrum of observed effects from prenatal alcohol exposure suggests that all types of FASD are not mediated by the same mechanism. Whether a single or cascading mechanism exists is a complex issue facing current FASD research (Abel and Hannigan, 1995; West et al., 1994). Until this issue can be resolved with more clarity, development of successful intervention strategies or therapeutics will be difficult.

3. EFFECT OF ALCOHOL ON OVINE FETAL HIPPOCAMPAL FORMATION AND OLFACTORY BULB

3.1 Introduction

Maternal alcohol exposure can lead to debilitating outcomes in the offspring that range from mild behavioral and learning disabilities to severe physical and mental deficits that are collectively termed Fetal Alcohol Spectrum Disorders (FASD) (Riley and McGee, 2005; Sokol et al., 2003). Serious behavioral problems and altered performance in learning and/or memory tasks in FASD children (for review, see Mattson et al., 2001) are thought to be caused by hippocampal injury (Uecker and Nadel, 1996). Further support for this conclusion is provided by Hamilton and coworkers (2003) who reported deficits in place learning and spared cue-navigation among FASD children, and by magnetic resonance images of hippocampus displaying morphologic left-right asymmetries in FASD children (Riikonen et al., 1999).

There exists no quantitative study of hippocampal neuron numbers in children prenatally exposed to alcohol. However, there are a number of studies utilizing animal models that have reported differences in the number of hippocampal neurons in alcohol exposed subjects and that have addressed the issue of when, relative to development, the hippocampus is vulnerable to prenatal alcohol exposure. West et al. (1986), utilizing the rat model reported a 10% increase in dentate gyrus granule cell number in response to third trimester-equivalent alcohol exposure. Similarly, Miller (1995), using rats, showed a blood alcohol concentration (BAC)-dependent change in dentate gyrus granule cell number, with an increase in cell number following moderate alcohol exposure and a

decrease in response to high BAC (third trimester-equivalent). However, the number of CA1 pyramidal cells was decreased in response to moderate doses of prenatal alcohol exposure, but no decrease in cell numbers was observed following exposure to the same BACs during the period of third trimester equivalent (Miller, 1995). Moreover, Livy and coworkers (2003), also utilizing rats, demonstrated decreased numbers of granule cells, CA1, and CA3 pyramidal cells in response to high doses of alcohol (BAC, 339 mg/dl), but only when the exposure period included the third trimester-equivalent of human brain development, and Maier and West (2001b) demonstrated no reductions in hippocampal cell numbers when the alcohol administration period was limited to the first two trimester-equivalents. In conclusion, it has been demonstrated in the rat model that the hippocampus is vulnerable to prenatal alcohol exposure and that the third trimester exposure is required for injury.

While the hippocampus has long been known to be important in spatial learning and memory, recent research has focused on the contributions of extrahippocampal regions to spatial learning and memory. Lesions of the entorhinal cortex and olfactory bulb lead to deficits in spatial working memory and reversal learning. The piriform, perirhinal and lateral enterorhinal cortical areas receive common excitatory inputs from the olfactory bulb and are interconnected to form a hippocampal circuit implicated in modulating memory (Obernier et al., 2002). The olfactory bulb undergoes neurogenesis at the same time as the cerebellum, and it has been well established that the cerebellum is sensitive to alcohol exposure (Maier et al., 1999). A permanent decrease in the number of mitral cells in the olfactory bulb has been demonstrated in the rat model as a result of

alcohol exposure (Barron and Riley, 1992; Bonthius et al., 1992; Bonthius and West, 1991a, 1991b). In addition, Pieffer et al. (Peiffer et al. (1979) observed olfactory bulb agenesis and morphological defects of the olfactory stalks in their report on human alcohol teratology. Early olfactory learning in rodents is also used as a model to study neurobehavioral deficits due to prenatal alcohol exposure (Kirstein et al., 1997). Considering the importance of the olfactory bulb in various leaning and memory tasks, it renders this unique brain region a relevant target for investigation.

The purpose of this study was to test the hypotheses that prenatal alcohol exposure alters development of the hippocampal formation and the olfactory bulb in an ovine model, where all three trimester equivalents of human brain development occur prenatally as they do in humans. The maximum velocity of brain growth occurs at the time of parturition in humans, whereas it occurs postnatally in rats (Dobbing and Sands, 1973, 1979), requiring the assessment of the response to alcohol administration during the third trimester-equivalent of human brain development in rats to be conducted postnatally. Therefore, to extrapolate the findings from neonatal rats to humans, one must assume that the intrauterine environment, placenta, and maternal interactions do not play an important role in mediating the damage (Cudd, 2005; Ramadoss et al., 2007b). In order to more closely mimic alcohol exposure in humans, the sheep model was selected in this study; in sheep, all three trimester equivalents of human brain development occur prenatally (Dobbing and Sands, 1979). It is hypothesized that maternal alcohol binging during all three trimester-equivalents would result in reduced cell numbers in the hippocampal formation and olfactory bulb. This study specifically

examined the effect on the pyramidal cells of the CA1, CA 2/3 subfields, the granule cells of the dentate gyrus and the mitral cells of the olfactory bulb. A weekend binge pattern, a drinking pattern common in women who use alcohol during pregnancy, was selected for the experimental protocol of alcohol administration (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a; Ramadoss et al., 2007a). Further, since it is reported that all three trimester alcohol exposure at a dose of 1.75 g/kg resulted in cerebellar neuronal loss in fetal sheep, we utilized the same dose for studying hippocampal and olfactory neuronal deficits in this study (Ramadoss et al., 2007b).

3.2 Methods

Animals and Breeding

In this study, fetuses of 12 adult pregnant ewes were utilized. The experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI, 20 mg) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on 25, 60 and 90 days to confirm pregnancy. On day 4 of gestation,

the saline control and alcohol group subjects were moved into individual pens but were able to see herd mates in the adjacent pens at all times. Conditions of constant temperature (22 ^{0}C) and fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX).

Alcohol Dosing Protocol

Two treatment groups, an alcohol group (alcohol dosage of 1.75 g/kg body weight) and a saline control group that received 0.9% saline of a volume and at an infusion rate equivalent to that of the alcohol dose, were studied. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% w/v in sterile saline administered over one hour. Alcohol was administered on three consecutive days followed by four days without alcohol beginning on day 4 of gestation and continuing until day 132 of gestation which corresponds with the end of the third trimester of human brain development, the peak of the brain growth spurt in sheep.

Experiment Protocol

Details of the experimental protocol have been described elsewhere (Ramadoss et al., 2006). In brief, the experiments began on day 4 and were terminated on day 133 of gestation. On gestational day 4, an intravenous catheter (16 gauge, 5.25 in Angiocath^{TM} Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein. On the day of infusions, ewes were connected to the infusion pump by 0830 hr and

alcohol was infused continuously over 1 hr, between 0830 and 0930 hr. On gestational day 42, after pregnancy was confirmed ultrasonographically, the ewes underwent surgery to chronically implant femoral arterial and venous vascular access ports (V-A-P^{TM}, Model CP 47P, Access Technologies, Skokie IL). The arterial vascular access port was implanted to monitor maternal blood gas status. The ewes were not surgically instrumented until after the first trimester to avoid early embryonic losses. Infusions were then given through the venous port and blood was sampled from the arterial port.

Blood Alcohol Concentration (BAC) Measurement

Blood was drawn from the jugular vein catheter on gestational days 6 and 40 and from the femoral artery catheter on days 90 and 132 one hour following the commencement of alcohol infusions for the measurement of BAC. A 20 µl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Fetal Hippocampal and Olfactory Bulb Tissue Processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline

followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

The fetal brain was divided parasagittally and the right olfactory bulb and hippocampus were removed. The tissues were then dehydrated through increasing concentrations of alcohol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin™ Embedding kit, Leica, Wetzlar, Germany). Following infiltration, the tissue was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned coronally into 30 μm sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were mounted serially on glass slides, stained with cresyl violet, and coverslipped. A representative coronal section from a normal control brain is presented in Figure 1.

Stereological Cell Counting

The total number of fetal hippocampal pyramidal cells in the CA1, CA2/3 fields, the granule cells in the dentate gyrus, and the mitral cells in the olfactory bulb were estimated using unbiased stereological cell counting techniques as described in previous publications (West et al., 2001). The term CA2/3 refers to the summed CA2 and CA3 fields, combined due to the difficulty in differentiating these two fields accurately at this developmental stage. The Nikon (Garden City, NY) Optiphot microscope used in this study had a 4X objective lens for volume measures and a 60X objective lens with a 1.4

numerical aperture condenser for density measures. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \times A(p_i) \times t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma disector \times A(fr) \times h)$ where ΣQ is the sum of the hippocampal/olfactory cells counted from each disector frame, $\Sigma disector$ is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of cells in the olfactory bulb, dentate gyrus, CA1, and the CA2/3 fields were then calculated by multiplying the reference volume of the respective regions and the numerical density of cells within this reference volume as described before (West et al., 2001).

Data Analysis

Data are presented as mean ± SEM. All stereology measures were analyzed using unpaired two-tailed t tests to compare between the control and alcohol groups.

Statistical significance and statistical trends were established *a priori* at < 0.05 and < 0.10 respectively.

3.3 Results

No differences were seen in fetal body and brain weights between groups. The mean BACs measured on days 6, 40, 90 and 132 of gestation for alcohol group peaked at 1 hour which coincided with the end of infusion. The BACs on the different days for alcohol group did not differ significantly and were therefore combined. The mean peak BAC was 189 $\pm$ 19 mg/dl. Subjects remained conscious throughout and after the alcohol infusion but appeared ataxic if encouraged to walk shortly after the end of the infusion. As reported previously, we observed maternal hypercapnea, acidemia, and normoxemia with every bout of alcohol (Cudd et al., 2001a; Ramadoss et al., 2007b).

Stereology Data

The density of dentate gyrus granule cells trended higher by 16% in alcohol exposed fetuses ($t_{10} = -1.97$, $p = 0.0773$) (Figure 2). No such differences were found for the pyramidal cells of the CA2/3 field ($t_{10} = -1.56$, $p = 0.1488$), the CA1 field ($t_{10} = -1.44$, $p = 0.1804$), and the mitral cells of the olfactory bulb ($t_{10} = -0.115$, $p = 0.9107$) between groups.

The volume of the dentate gyrus trended lower in the all three trimester binge alcohol group by 14% ($t_{10} = 1.89$, $p = 0.0883$) (Figure 3). The reference volume for the CA2/3 field ($t_{10} = 1.35$, $p = 0.2078$), CA1 field ($t_{10} = 0.128$, $p = 0.9006$) and the mitral cells ($t_{10} = 1.5$, $p = 0.1656$) were not different between groups.

Unlike the density and the volume measures, the estimated mean total number of fetal dentate gyrus granule cells was not different among groups ($t_{10} = 0.129$, $p = 0.8998$) in response to maternal alcohol binging throughout gestation (Figure 4). No differences were found in the total cell number for CA2/3 pyramidal cells ($t_{10} = 0.0354$, $p = 0.9725$), CA1 pyramidal cells ($t_{10} = -0.828$, $p = 0.4271$), and the mitral cells ($t_{10} = 1.56$, $p = 0.1501$) between groups.

3.4 Discussion and Conclusions

Effects of All Three Trimester-equivalent Alcohol Exposure on the Ovine Fetal Hippocampal Formation

A moderate dose of alcohol creating BACs of 189 mg/dl administered in three consecutive day per week binges throughout gestation selectively targeted the dentate gyrus. Total dentate gyrus cell neuron numbers were unchanged while the density was increased by 16% ($p = 0.0773$) and the volume was decreased by 14% ($p = 0.0883$) making the case that development was altered by prenatal alcohol exposure. Density, reference volume, and the number of pyramidal cells in the CA1 and CA2/CA3 fields were not altered by prenatal alcohol exposure. The selective effect of prenatal alcohol exposure on the dentate gyrus is in agreement with studies performed in the rat model. West and coworkers (1986) demonstrated that third trimester-equivalent exposure using a higher dose of alcohol (BAC 380 mg/dl) produced a 10% increase in the density of granule cells in the dentate gyrus, but observed no change in the density of CA1 or CA3 pyramidal cells. Miller (1995) demonstrated an increase in dentate gyrus granule cell number in postnatal rat pups in response to moderate alcohol exposure (BAC, 132

mg/dl), and a decrease in their number in response to BACs of 339 mg/dl. Further, first two trimester-equivalent alcohol exposure did not alter the cell number. Similarly, Livy and coworkers (2003) showed decreased granule cell numbers in response to a high BAC of 339 mg/dl, but only when the exposure period included the third trimester-equivalent. Maier and West (2001b) showed that none of the hippocampal cell types were altered in response to alcohol exposure limited to the first two trimester-equivalents. Thus, we and others have shown an increase in density and/or number in response to moderate alcohol exposure and a decreased number in response to higher BACs during the third or all three trimester-equivalents, but none of these alterations are observed when the exposure period does not include the third trimester-equivalent.

Our study also demonstrates no alteration in the total number of hippocampal CA1 and CA2/3 field pyramidal cell numbers in response to all three trimester-equivalent binge alcohol exposure in fetal sheep. A careful comparison with the neuroanatomical studies conducted using the rat model reveals that these findings are not different from earlier reports. Miller (1995) demonstrated no reductions in CA1 pyramidal cell number in response to third trimester-equivalent alcohol (BAC, 222 mg/dl) and decreases at much higher does (BAC, 339 mg/dl). Similarly, Bonthius and West (1990) demonstrated decreased CA1 pyramidal cells in response to postnatal alcohol exposure at the highest dose (BAC, 361 mg/dl), while no such reduction was found in response to a BAC of 190 mg/dl. The more recent study by Livy and coworkers also demonstrated decreased number of CA1 and CA3 pyramidal cells in response to high doses of alcohol (BAC, 339 mg/dl), but these effects were again not observed when

the exposure period did not include the third trimester-equivalent (Livy et al., 2003). These findings are further supported by another study where first two trimester-equivalent alcohol exposure did not result in any difference between the alcohol and the control groups (Maier and West, 2001b). Taken together, all three trimester-equivalent exposure to moderate doses of alcohol does not alter the number of pyramidal cells in the developing hippocampus, whereas exceptionally high BACs may selectively reduce CA1 pyramidal cell number. Interestingly, we recently reported that the estimated number of cerebellar Purkinje cells were reduced in response to all three trimester-equivalent alcohol exposure (BAC, 189 mg/dl) in the sheep model (Ramadoss et al., 2007b), a finding when taken together with this report demonstrates the regionally selective actions of alcohol on the fetal brain.

Effects of All Three Trimester-equivalent Alcohol Exposure on the Ovine Fetal Olfactory Bulb

Similar to our findings from the evaluation of hippocampal pyramidal cells, there was no change in the number and the density of mitral cells in the fetal sheep olfactory bulb. Additionally, the reference volume of mitral cells was not affected by the all three-trimester equivalent alcohol exposure. Findings derived using a rat model and third-trimester equivalent alcohol exposure paradigm show reduction in mitral cell number (cell density did not change, but reference volume was reduced) in response to alcohol treatment (Maier et al., 1999). Interestingly, in this same study by Maier and coworkers, there were no changes in mitral cell numbers if the alcohol was administered throughout all three trimester equivalents. This is consistent with our findings of no change in mitral

cell number, density or reference volume in response to all three trimester exposure in the sheep. The lack of change in mitral cell number does not exclude the possible presence of functional abnormalities, alterations in neurotransmitter or neurotrophic factor levels, or changes in olfactory bulb granule cell numbers or function. Further exploration of the possibility of functional but not structural injury in response to prenatal alcohol exposure is warranted.

We conclude that consumption of a moderate dose of alcohol *in utero* throughout gestation selectively targets the dentate gurus of the fetal hippocampal formation. We also demonstrate that a moderate dose of alcohol administered throughout gestation does not alter the CA2/3 and CA1 pyramidal cell numbers in the fetal hippocampus and the mitral cells in the olfactory bulb. The significance of these findings is that FASD children exhibit behavioral deficits that include altered performance in tasks such as learning and memory, tasks that involve hippocampal function (Mattson et al., 2001). Children exposed to alcohol prenatally exhibit hippocampus-associated impaired learning (Hamilton et al., 2003), and memory deficits (Uecker and Nadel, 1996), and these impairments have been correlated to hippocampal structural deficits (Riikonen et al., 1999). These findings further establish, using an animal model that closely models human development and prenatal alcohol exposure, that the hippocampus is an important target of prenatal alcohol exposure and provides support that third trimester equivalent of human brain development exposure is required for injury.

4. ROLE OF CORTISOL IN CHRONIC BINGE ALCOHOL-INDUCED CEREBELLAR INJURY IN SHEEP

4.1 Introduction

Prenatal alcohol exposure has been demonstrated to result in elevated cortisol levels in infants (Jacobson et al., 1999), neonatal rats (Weinberg, 1989) and fetal sheep (Cudd et al., 2001b). While normal cortisol concentrations are vital for normal brain development and somatic growth, increases in cortisol during development could interfere with neuronal growth, proliferation and differentiation, expression of neuronal and glial antigens and myelination (Bohn, 1984). High levels of glucocorticoids have been shown to cause an alteration in hippocampal development and learning ability (Bodnoff et al., 1995).

Glucorticoid administration to women undergoing preterm labor, or directly to preterm infants has been widely used between the 24^{th} and 34^{th} week of gestation to help reduce the incidence of respiratory distress in preterm infants (Gilstrap, et al., 1995). Growing evidence indicates that the administration of glucocorticoids during the perinatal period can result in long-term liabilities including altered brain architecture (McGowan, et al., 2000; Murphy et al., 2001) and behavioral and cognitive deficits (Jameson et al., 2006). Similarities in brain responses have been noted between fetal alcohol exposure and perinatal exposure to high glucocorticoid levels, suggesting that an alteration in maternal and/or fetal hypothalamus-pituitary-adrenal (HPA) axis activity and glucocorticoid levels may be responsible in part for the neurobehavioral deficits

seen with prenatal alcohol exposure (Kim et al., 1999, Weichsler, 1977; Weinberg, 1989).

Previous reports from our laboratory have shown that third trimester equivalent binge alcohol exposure at a dose of 1.75 g/kg results in significant cerebellar Purkinje cell loss in fetal sheep (Ramadoss et al., 2007a; 2007b; West et al., 2001) and also results in a significant increase in both maternal and fetal adrenocorticotropin (ACTH) and cortisol levels (Cudd et al., 2001b; Ramadoss et al., 2008b). In this study, we hypothesized that repeated elevations in cortisol from chronic binge alcohol exposure are responsible, at least in part, for fetal neuronal deficits.

4.2 Methods

Animals and Breeding

Pregnant ewes were divided in to 4 groups: normal control, saline control, alcohol and cortisol, with 5 sheep per group. The experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI, 20 mg) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed

ultrasonographically to confirm pregnancy. Conditions of constant temperature (22 ^{0}C) and fixed light dark cycle (12:12) were maintained. Subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX).

Alcohol and Cortisol Dosing Protocol

The alcohol group received an alcohol dosage of 1.75 g/kg body weight in 0.9% saline of a volume and at an infusion rate equivalent to that of the alcohol dose, administered to the saline control and cortisol group. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% w/v in sterile saline administered over one hour. Infusions were administered on three consecutive days followed by four days without alcohol beginning on day 109 of gestation and continuing until day 132 of gestation. The magnitude of elevation in maternal cortisol in response to alcohol (Cudd et al., 2001b) was mimicked in the cortisol group by infusing hydrocortisone for 6 hours on each day of the experiment. Determination of the cortisol infusion rate was based on the metabolic clearance rate for cortisol in sheep (Wood, 1987), and empirical pilot data verifying that the resultant plasma cortisol values were like those resulting from the alcohol infusion paradigm. The infusion rate to create levels matching that seen in response to alcohol (Cudd et al., 2001b) was 0.8 µg/kg/min for the first two hours, followed by 0.4 µg/kg/min for hours 3 through 6. Solu-Cortef® (hydrocortisone sodium succinate for injection, USP) was diluted in 0.9% saline and administered via a syringe pump (Harvard Apparatus syringe pump Model 964).

Experiment Protocol

In brief, the experiments began on gestational day (GD) 109 and were terminated on GD 133. On gestational day 104, the ewes underwent surgery to chronically implant maternal femoral arterial and venous vascular access ports (V-A-P^{TM}, Model CP 47P, Access Technologies, Skokie IL). On the day of infusions, ewes were connected to the infusion pump by 0830 hr and alcohol or saline was infused continuously over 1 hr, between 0830 and 0930 hr. In the cortisol group, the magnitude of the maternal elevation of cortisol in response to alcohol was mimicked by infusing hydrocortisone for 6 hours. Infusions were given through the venous port and blood was sampled from the arterial port.

Blood Alcohol Concentration (BAC) Measurement

Blood was drawn from the jugular vein catheter on GD 109 and from the femoral artery catheter on GD 116, 123 and 132 one hour following the commencement of alcohol infusions for the measurement of BAC. A 20 µl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Fetal Cerebellar Tissue Processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

The cerebellum was removed and the tissues were then dehydrated through increasing concentrations of alcohol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin^{TM} Embedding kit, Leica, Wetzlar, Germany). Following infiltration, the tissue was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned coronally into 30 μm sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were mounted serially on glass slides, stained with cresyl violet, and coverslipped.

Stereological Cell Counting

The total number of Purkinje cells in the cerebellum were estimated using unbiased stereological cell counting techniques as described in previous publications (West et al., 2001). The Nikon (Garden City, NY) Optiphot microscope used in this study had a 4X objective lens for volume measures and a 60X objective lens with a 1.4 numerical aperture condenser for density measures. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis.

The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \times A(p_i) \times t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma \text{disector} \times A(fr) \times h)$ where ΣQ is the sum of the hippocampal/olfactory cells counted from each disector frame, Σdisector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of Purkinje cells in the cerebellum was then calculated by multiplying the reference volume of the respective regions and the numerical density of cells within this reference volume as described before (West et al., 2001).

Statistical Analysis

Data are presented as mean ± SEM. Maternal plasma cortisol levels were subjected to a two way analysis of variance with time point as the "within" factor and treatment group as the "between" factor. Fetal weight data and Purkinje cell number were subjected to a one way analysis of variance with treatment group as the sole independent variable. Significant main effects or interactions were subjected to multiple

comparison testing with Fisher's least significant difference test. Statistical significance and statistical trends were established *a priori* at $p = < 0.05$ and < 0.10 respectively.

4.3 Results

The mean blood alcohol concentration in the alcohol group was 239 ± 7 mg/dl.

Fetal cerebellar Purkinje cell number (Figure 5) differed significantly among groups, with alcohol differing from saline and normal control groups ($p = 0.008$ and 0.038 respectively). The cortisol group did not differ from the alcohol group ($p = 0.06$) nor the saline or normal control groups ($p = 0.259$ and 0.601 respectively). There were no significant differences among the saline and normal control groups.

Fetal weight and body length did not differ among groups ($p = 0.8$ and 0.89 respectively), but there was trend significance in fetal adrenal weight ($p = 0.09$) among groups, with the cortisol and alcohol group exhibiting a lower mean adrenal weight than the saline or normal controls (Figure 6).

Maternal plasma cortisol concentrations were significantly increased in the alcohol and cortisol group compared to the saline control group at 120 minutes (Figure7). The alcohol and cortisol groups did not differ significantly from each other at any time point. The mean maternal plasma cortisol levels were 12.8 ± 6 ng/ml, 25.4 ± 6 ng/ml, and 45.8 ng/ml ± 11 at 0, 1 and 2 hours respectively.

4.4 Discussion and Conclusions

In animals that give birth to mature young (such as sheep, primates, and guinea pigs) maximal brain growth and a large part of neuroendocrine maturation takes place *in utero* (Matthews, 2000). In contrast, in species that give birth to immature young (such

as rats, mice, and rabbits), much of endocrine development occurs in the postnatal period (Matthews, 2000). Since all three trimesters occur *in utero* in the sheep, it more closely models human brain and neuroendocrine development and is an appropriate animal model choice for this experiment.

The placenta presents a complete barrier to ACTH (Jones, et al., 1975) and a partial barrier to cortisol (Bietens et al., 1970), with estimates of approximately 10-20% of maternal cortisol crossing the placenta to the fetus in humans (Murphy, et al., 2006, Gitau et al., 1998). In human pregnancy, endogenous maternal cortisol concentrations are 5-10 times higher than fetal cortisol concentrations. Placental 11 β-hydroxysteroid-dehydrogenase activity, an enzyme that converts cortisol to an inactive metabolite, maintains this maternal: fetal difference and protects the fetus from the high maternal concentrations of endogenous glucocorticoids (Murphy et al., 2006). However, since fetal concentrations of cortisol are much lower than maternal concentrations, a contribution of 10-20% from the mother could still double fetal concentrations (Murphy, et al., 2006, Gitau et al., 1998) and maternal cortisol concentrations have been reported as accounting for as much as 40% of the variance in cortisol concentration in the human fetus (Gitau et al., 1998). Therefore, administration of cortisol maternally, as administered in this study, can reasonably be expected to increase fetal cortisol levels.

While concurrent measurement of both maternal and fetal cortisol levels would have been ideal in this study to confirm and more accurately compare the fetal exposure level, fetal surgery would have had to be performed during the study. We chose to avoid any potential confound of surgery and instrumentation during the experiment on cortisol

concentrations and fetal cerebellar Purkinje cell number. The fetus cannot be instrumented before GD 113, which is during the third trimester equivalent and thus during the time of this experiment (GD 109-133). Maternal and fetal plasma cortisol levels have been shown to have a linear correlation (Gitau et al., 1998) and both maternal and fetal cortisol concentrations in response to alcohol have been characterized in the sheep model (Cudd et al., 2001b).

Maternal alcohol consumption during pregnancy could alter fetal HPA activity by crossing the placenta and directly activating the fetal HPA axis, which is functional before birth, or alcohol could act indirectly through effects on maternal pituitary-adrenal function (Weinberg and Bezio, 1987). Studies in pregnant ewes in the third trimester equivalent have demonstrated a stimulatory action of alcohol on both the maternal and fetal HPA axis (Cudd et al., 2001b).

The human fetal adrenal gland synthesizes cortisol *de novo* after the 28th week (beginning of third trimester) of pregnancy, which means the fetus is dependent on maternal cortisol for approximately the first 70% of pregnancy (Mastorakos, 2003). In fetal sheep, the source of fetal cortisol is almost entirely maternal until GD 121 (the first 82% of pregnancy) and then, like the human, fetal adrenal responsiveness begins to change and fetal production increases. Fetal production is responsible for approximately 1/3 of the fetal cortisol concentration between GD 122-133 (Beitins et al., 1970; Hennesy et al., 1982); the third trimester equivalent in sheep (which is the time period of this experiment) is GD 109-133 (Figure 8). After GD 133 in the sheep, the source of cortisol is almost exclusively of fetal origin. Likewise, in the human, cortisol levels

triple late in pregnancy and undergo a further abrupt rise in association with labor (Murphy et al., 2006).

Though this experimental design closely mimics the increases in cortisol seen in response to alcohol, there is a possibility that the fetal cortisol group and fetal alcohol groups did not have identical cortisol concentrations. This study utilized a third trimester equivalent exposure pattern in sheep (GD 109-133) and during this time, fetal adrenal responsiveness changes and approximately 1/3 of fetal cortisol is from fetal production on GD 121-133 (Beitins et al., 1970; Hennesy et al., 1982). Increases in maternal plasma cortisol concentration have been shown to increase fetal plasma cortisol levels and this in turn can provide negative feedback for fetal ACTH (Wood, 1987). Therefore during GD 121-133 it is possible that the increase in maternal plasma cortisol concentration in the cortisol group could have lead to negative feedback in fetal ACTH levels, potentially lowering total fetal plasma cortisol concentration. This negative feedback would also occur in the alcohol group, but it has been demonstrated that alcohol also stimulates the fetal HPA axis (Cudd et al., 2001b) and this could override the ACTH inhibition. Thus, the net result could be differences in fetal plasma cortisol concentrations in the alcohol and cortisol groups. However, the trend decrease in adrenal weight in the cortisol group suggests that they had did indeed have high fetal plasma cortisol levels. Future experiments comparing prenatal steroid administration and alcohol exposure could be strengthened by measuring fetal cortisol and ACTH plasma concentrations, however as mentioned previously this adds the potential confounding factor of surgery and instrumentation on Purkinje cell number.

In summary, our data demonstrates that repeated elevations in maternal glucocorticoids may not independently produce fetal cerebellar Purkinje cell loss during the brain growth spurt. However, the elevations in cortisol along with other changes induced by alcohol may together mediate brain injury in FASD.

5. AVAILABILITY OF GLUTAMINE AND RELATED AMINO ACIDS IN OVINE FETUS IN RESPONSE TO ACUTE ALCOHOL EXPOSURE OR ACUTE ACIDEMIA

5.1 Introduction

A key diagnostic feature of FAS is growth retardation (Sokol and Clarren, 1989). In children, prenatal alcohol exposure has been associated with growth retardation that can persist into adolescence (Day et al., 2002) and low birth weight can lead to altered development and programming, which can have lifelong consequences (Barker, 1994; Wu et al., 2004). The fetus depends on steady supply of nutrients for growth and development, and disturbances in this supply can have the detrimental effect of impaired development and growth retardation.

The mechanisms for these growth deficits that occur with prenatal alcohol exposure are not well understood; there are three possible ways that the fetal nutrient supply could be disrupted. First, alcohol could lead to fetal undernutrition by reducing maternal dietary intake (Schenker et al., 1990) and fetal growth is vulnerable to maternal protein deficiency (Wu et al., 1998). Detrimental effects from prenatal alcohol exposure have been documented when maternal nutrition was adequate, suggesting maternal malnutrition is not obligatory for the development of alcohol-induced fetal damage (Schenker et al., 1990). This may be explained by the recent findings that a chronic third trimester binge alcohol pattern of exposure reduced the concentration of multiple amino acids in the maternal plasma of ewes on adequate nutrition (Ramadoss et al., 2008c). Second, alcohol has been shown to alter the placental transport and/or

metabolism of nutrients, therefore depriving the fetus of nutrition (Fisher et al., 1981; Lin et al., 1981; Henderson et al., 1981). And third, an alteration in maternal and/or fetal metabolism and compartmentalization of nutrients could have a net result of fetal undernutrition (Schenker et al, 1990; Ramadoss et al., 2008c).

Amino acids are not only building blocks for proteins, but are also signal molecules and key regulators of metabolic pathways (Wu, 2009). A reduction of multiple amino acids in response to prenatal alcohol exposure has been documented in the rodent model. Maternal plasma concentrations of threonine, serine, glutamine, glycine, alanine, and methionine were reduced in response to acute alcohol exposure in the mouse (Padmanabhan, et al., 2002). Chronic alcohol exposure in the rat reduced maternal plasma proline concentrations (Marquis et al., 1984) and increased fetal plasma glutamate concentrations (Karl et al., 1995). In the sheep model, a chronic third trimester binge alcohol exposure paradigm reduced the maternal plasma amino acid concentration of arginine, citrulline, glutamine, asparagine, serine, threonine, tryptophan, methionine, leucine, histidine, tyrosine, valine and isoleucine, while glutamate was increased (Ramadoss et al., 2008c).

Most of this information characterizes the effect of alcohol on maternal amino acids, while there is a paucity of information on the effect to the fetus. This study exploits the unique strengths of the sheep model, in that pregnant ewes and their fetus can be instrumented to allow simultaneous sampling of maternal and fetal blood in order to investigate and compare the changes in response to alcohol administration. It provides information on maternal amino acid alterations in response to a single acute

alcohol exposure (analogous to one binge episode) and allows comparison of maternal and fetal alterations.

Results of work by Ramadoss and colleagues support the conclusion that chronic binge alcohol-induced acidosis alters amino acid homeostasis in pregnant ewes through glutamine-dependent pathways (Ramadoss et al., 2008c). These maternal decreases in multiple amino acids impact the nutrient supply to the fetus and could lead to impaired growth and development. We hypothesize that the concentration of many of these same amino acids will also be reduced in the fetus as a result of decreased maternal bioavailability as well as an alcohol-induced impairment of placental transfer. For the first time, we will report the amino acid concentrations simultaneously in the mother and fetus, by sampling maternal and fetal blood at the same time in response to alcohol administration.

In addition, the candidate mechanism of alcohol-induced acidosis that governs the maternal alterations in amino acids was evaluated in the fetus. Creating the same magnitude and pattern of acidemia, independent of alcohol exposure, as that produced by alcohol caused plasma amino acid reductions that were similar to those seen in the alcohol group in a chronic exposure paradigm in pregnant ewes (Ramadoss, et al., 2008c). This study examined the response to a single acute dose of alcohol (analogous to a single binge episode) and a single acidemic challenge (independent of alcohol) of the same magnitude as that seen in the alcohol group. This allowed comparison of changes that occur in the maternal plasma amino acid concentrations with an acute

versus chronic exposure pattern. Further, fetal samples were collected to compare amino acid perturbations in the mother with plasma amino acid concentrations in the fetus.

Alcohol-induced acidemia has been proposed as the central mechanism governing the changes in maternal amino acid concentrations that occur with alcohol administration (Ramadoss, et al., 2008c). Disturbance in pH was hypothesized to be a mechanism underlying the teratogenic effects of alcohol even before FAS was widely recognized or characterized (Horiguchi et al., 1971; Jones et al., 1973). A mixed respiratory and metabolic acidosis occurs in humans in response to alcohol consumption, and the drop in blood pH is directly proportional to the blood alcohol concentration (Zehtabchi et al., 2005; Lamminpaa and Vilska, 1991; Sahn et al., 1975). Transient increases in the arterial partial pressure of carbon dioxide, resulting in a reduction of both maternal and fetal arterial pH occur with every bout of alcohol exposure (Cudd et al., 2001a; Ramadoss et al., 2006, 2007a, 2007b; West et al., 2001).

Acute pH changes are known to alter glutamine/glutamate metabolism (Curthoys and Watford, 1995; Nissim, 1999). The metabolism and inter-organ fluxes of glutamine in the sheep resemble that seen in humans (Heitman and Bergman, 1980). The dynamics of the maternal amino acid changes observed with alcohol or acidemia can be explained by glutamine dependent pathways that effect maternal amino acid homeostatic responses to alcohol or acidemia (Ramadoss et al., 2008c). Maternal glutamine concentrations decreased by approximately 40 percent in response to alcohol (Ramadoss, et al., 2008c) and we hypothesize that this decrease will be reflected in fetal plasma.

Glutamine is a major nutrient required by the fetus for growth (Kwon et al., 2003). It is an abundant amino acid in the ovine allantoic and amniotic fluid and is essential for the synthesis of nucleotides, NAD(P)+, and aminosugars (Kwon et al., 2003). Glutamine also plays an important role in fetal nitrogen and carbon metabolism (Vaughn et al., 1995). It is important to note that glutamine is the precursor for the brain neurotransmitter glutamate, which is likewise used for the biosynthesis of the cellular antioxidant glutathione (Mates, et al., 2002). Glutamine has also been implicated as having an important role as an apoptosis suppressor (Mates et al., 2002). Compared to all other amino acids, glutamine is the most versatile (Neu, 2001) and it is a precursor for the synthesis of other amino acids, including ornithine, citrulline, and arginine (Wu and Morris, 1998). Since it is evident that glutamine has a role in several processes that are disrupted by alcohol, we hypothesize that an acute administration of glutamine may have a protective effect. The effect of an acute administration of glutamine given to the ewe, concurrent with alcohol administration, was tested in this study to evaluate its potential as an intervention strategy.

In summary, this study addresses 5 major questions: 1) Is the same pattern of maternal amino acid disturbances demonstrated in the chronic exposure pattern also present with a single acute challenge of alcohol or acidemia? 2) Are the changes seen in maternal amino acid concentrations also present in the fetus? 3) The maternal response to alcohol and acidemia are similar, will this be true in the fetus too? 4) Will an acute administration of glutamine (given to the ewe) prevent the maternal and/or fetal fall in pH seen with alcohol administration? 5) Will an acute administration of glutamine

prevent the fall in maternal amino acid concentrations seen with alcohol administration (and how will it affect the fetus)?

5.2 Methods

Animals

The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Pregnant suffolk ewes aged 2-6 years with known date of conception (pregnancy was confirmed by ultrasound) were divided into six groups. A pair-fed instrumented control group contained ewes which were individually pair-fed according to consumption patterns of individual ewes in the alcohol group. This group was surgically cannulated and given physiological saline in a dose that was isovolumetric to the alcohol dose to control for manipulations associated with cannulation and infusions. The alcohol group received a 1.75 g/kg alcohol infusion. In the acidemia group, the maternal pH was manipulated to create an acidemia of the same magnitude as that caused by alcohol in previous studies (Cudd et al., 2001a; Ramadoss et al., 2008b, 2008c). This was achieved by altering inspired CO_2 concentration. Alcohol + glutamine dosed at 30 mg/kg and alcohol + glutamine dosed at 100 mg/kg groups received an intravenous bolus of glutamine at the beginning of the experiment when the alcohol infusion was started in order to test the ability of two different doses of glutamine to prevent the alcohol mediated fall in glutamine concentrations and/or the alcohol-induced fall in arterial pH. A glutamine control group received a dose of saline that was isovolumetric to the saline control and alcohol groups, as well as a single bolus of glutamine dosed at 30 mg/kg. Glutamine was infused

into the mother and not into the fetus as it is difficult to envision a practical protective/ameliorative strategy that would allow direct administration to a fetus and not have to employ provision of glutamine to the fetus via the mother. All groups consisted of 8 subjects and ewes were age and weight matched as closely as possible and assigned randomly to groups.

All animals were fed 4 lb/day of a "complete" ration (TAMU Ewe Ration, Nutrena). All animals consumed all of the feed offered. Food was withheld the morning of the experiment.

Experiment Protocol

In brief, the ewes and fetuses were surgically instrumented on gestational day (GD) 115±2 and the experiment was performed on GD 128±3, during the third trimester equivalent, a period of high vulnerability (Cudd, 2005). Blood samples from the mother and fetus were collected at 0 and 60min (the time of peak blood alcohol concentration) from the beginning of the treatment in order to examine the changes in both maternal and fetal arterial pH, pCO_2, O_2, and amino acid concentrations that occur during this time. Blood alcohol concentration (BAC) was measured at 60 minutes in the subjects receiving alcohol (the end of infusion).

Instrumentation

On day GD 115±2, pregnant sheep were anesthetized and aseptic surgery was performed Anesthesia was induced by using diazepam (0.2 mg/kg intravenously, Abbott Laboratories, North Chicago,IL) and ketamine (4 mg/kg intravenously,

Ketaset®, Fort Dodge, Fort Dodge, IA). The ewes were intubated endotracheally and ventilated spontaneously, and anesthesia was maintained by using isoflurane (0.5–2.5%, IsoFlo®, Abbott Laboratories) and oxygen. A ventral midline laparotomy was performed and the uterus was externalized and incised and a fetal hind limb was exteriorized. An incision was made over the craniolateral aspect of the hind limb midway between the hock and stifle. A catheter (0.030" inner diameter, 0.050" outer diameter polyvinyl chloride) was advanced from the cranial tibial artery into the abdominal aorta to the level of the diaphragm. The fetus was returned to the uterus and the uterus and maternal midline were closed. Catheters (0.050" inner diameter, 0.090" outer diameter polyvinyl chloride) were advanced from the maternal femoral artery and vein to the level of the diaphragm of the abdominal aorta and vena cava, respectively. Fetal and maternal catheters were passed through the abdominal wall in the flank region, where they were stored in a pouch attached to the skin. The average duration of surgery was 2 hours. At the conclusion of anesthesia, ewes were given a single injection of flunixin meglumine (1.1 mg/kg intramuscularly, Banamine®, Schering-Plough, Union, NJ), a prostaglandin synthase inhibitor, to reduce postoperative pain. Animals were treated postoperatively for 5 days twice daily with ampicillin trihydrate (25 mg/kg subcutaneously; Polyflex®, Aveco, Fort Dodge, IA) and gentamicin sulfate (2 mg/kg intramuscularly; Gentavet® 100, Velco, St. Louis, MO).

Alcohol and Glutamine Dosing Protocol

Infusions in the groups receiving alcohol were given at a dose of 1.75 g/kg body weight of a 40% w/v solution of alcohol in sterile saline administered over one hour. All

other groups received 0.9% saline of a volume and at an infusion rate equivalent to that of the alcohol dose. Infusion solutions were delivered intravenously using a Grady Medical VetFlo® 7701B IV automated infusion pump.

L-Glutamine powder from Sigma Aldrich (Cat #5792) was reconstituted with sterile water at a concentration of 20 ml sterile water per gram of powder and passed through a 0.7 μm bacteriostatic filter. The solution was kept at room temperature and prepared no sooner than 1 to 2 hours prior to administration. The dosage of glutamine was 30 mg/kg of body weight in the glutamine control and alcohol + glutamine at 30 mg/kg group and 100 mg/kg of body weight in the alcohol + glutamine at 100 mg/kg group and was based on extrapolation from the human glutamine turnover rate of 350 μmol/h/kg body weight (Kuhn, 1999). The glutamine solution was given as an intravenous bolus at the 0^{th} hour.

Blood Alcohol Concentration (BAC) Measurement

Blood was drawn from the maternal femoral vein catheter one hour following the commencement of alcohol infusions for the measurement of BAC. A 20 μl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the

column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Collection of Blood Samples and Blood Gas Analysis

Blood samples for amino acid measurements were collected in chilled polystyrene tubes containing lithium heparin. Tubes were kept on ice until they were centrifuged at 4°C for 20 minutes at 3200 rpm. Plasma was then separated and stored in separate aliquots to avoid the need to assay refrozen samples. Plasma samples were stored at -80°C.

Blood samples for fetal and maternal blood gas analysis (0.5 ml) were drawn anaerobically into heparinized 3 ml syringes. Samples were capped and placed on ice until analysis. Blood gases were measured at 37°C using a blood gas analyzer (ABL 5; Radiometer, Westlake, OH).

Amino Acid Analysis

Plasma was acidified with 1 ml of 1.5 mol/l $HClO_4$ and then neutralized with 0.5 ml of 2 mol/l K_2CO_3. The supernatant was used for amino acid analysis by HPLC, as described in previous publications (Wu et al., 1997).

Manipulation of Maternal Blood Gases

Maternal blood gases were measured and controlled by varying maternal inspired gases. On the day of the experiment, ewes were placed in a modified metabolism cart so that the animal's head is inside a plexiglass chamber. A vinyl diaphragm attached to the open side of the chamber will be drawn around the animal's neck to isolate the atmosphere in the chamber from ambient air (Figure 9). Subjects in the acidemic group

were exposed to increased inspired fractional concentrations of CO_2 to create a similar magnitude and pattern of reduction in the arterial pH compared to that produced by alcohol in previous studies (Cudd et al., 2001a). The rate at which CO_2 is introduced into the chamber in the acidemic group is determined by monitoring maternal arterial pH; the CO_2 inflow rate was adjusted so that maternal arterial pH in the acidemic and alcohol groups will be matched over the duration of the experimental period. The percentage of oxygen and carbon dioxide in the chamber is measured using a gas monitor (oxygen, model S-3A; carbon-dioxide, model CD-3A, Applied Technologies, Pittsburgh, PA). Normoxemic conditions are maintained throughout the experiment in the acidemic group. Subjects in the other groups also had their heads inside a plexiglass chamber, but the chamber bottom was removed to allow them to breathe room air.

Statistical Analysis

The percentage change in the levels of the maternal and fetal amino acids at one hour (the end of the alcohol or saline infusion) compared to that at the 0 hour (baseline), were analyzed using a one-way analysis of variance with treatment group as the sole independent factor. Maternal and fetal arterial pH, pCO_2, and O_2 were analyzed using a mixed analysis of variance with treatment as between and time as a within factor. Post-hoc tests were conducted using Fisher's protected least-significant difference test. The α level was established a priori at $p<0.05$ for all analyses; p values between 0.05 and 0.1 were considered trends.

5.3 Results

Maternal Blood Gas Data

There was no statistical difference in maternal arterial pH among groups at the beginning of the experiment (time 0). There was a statistically significant reduction in maternal arterial pH (Figure 10) in the alcohol and acidemia groups as well as in both alcohol + glutamine groups compared to the saline and glutamine control groups at 60 minutes (the time of peak blood alcohol concentration). The saline control and glutamine control groups did not differ from each other ($p=0.561$), but did differ from all other groups. The alcohol and acidemia groups did not differ from each other ($p=0.282$), and neither differed from the alcohol + glutamine 30 mg/kg group ($p=0.304$). The acidemia and alcohol groups both differed from the alcohol + glutamine 100 mg/kg group ($p=0.017$ and 0.006 respectively).

There was no statistical difference in maternal pCO_2 among groups at the beginning of the experiment (time 0). There was a statistically significant increase in maternal pCO_2 (Figure 11) in the acidemia group compared to all other groups at 60 minutes ($p<0.001$). None of the other groups differed from each other.

The maternal pO_2 did not fall below normal in any group at any time (Table 1).

Fetal Blood Gas Data

There was no statistically significant difference among groups at the beginning of the experiment (time 0). There was a statistically significant reduction in the alcohol group fetal arterial pH (Figure 12) compared to the saline group at 60 minutes (the time of peak blood alcohol concentration). The saline group had a statistically significant

higher pH at 60 minutes than all other groups, except for the glutamine control group which exhibited no difference compared to saline (p=0.536). The acidemia group had a statistically significant lower pH than all other groups at 60 minutes. The glutamine control group did not differ from the alcohol group (p=0.183), the alcohol + glutamine 30 mg/kg group (p=0.346), or the alcohol + glutamine 100 mg/kg group (p=0.268). The alcohol group did not differ from the alcohol + glutamine 30 mg/kg group (p=0.546) or the alcohol + glutamine 100 mg/kg group (p=0.667). The alcohol + glutamine 30 mg/kg and alcohol + glutamine 100 mg/kg groups did not differ from each other (p=.545).

There was no statistical difference in fetal pCO_2 among groups at the beginning of the experiment (time 0). There was a statistically significant increase in fetal pCO_2 (Figure 13) in the acidemia group compared to all other groups at 60 minutes (p ranged from 0.003-0.005). None of the other groups differed from each other.

The fetal pO_2 did not fall below normal in any group at any time (Table 1).

Maternal Plasma Concentration of Amino Acids

There was a statistically significant difference in the percent change from 0 to 60 minutes among groups for the following amino acids: aspartate, glutamate, asparagine, serine, glutamine, histidine, threonine, citrulline, arginine, methionine, valine, phenylalanine, isoleucine, leucine, and branched chain amino acids. Trends were present for glycine and tryptophan. The mean values are reported in Table 2 and depicted on Figures 14 and 15, with the details of which groups were statistically different for each amino acid summarized in Table 3.

Fetal Plasma Concentration of Amino Acids

There was a statistically significant difference in the percent change from 0 to 60 minutes among groups for the following amino acids: asparagine, glutamine, glycine, citrulline, arginine, alanine, tryptophan, methionine, valine, phenylalanine, isoleucine, ornithine and branched chain amino acids. Trends were present for glutamate, threonine, tyrosine and leucine. The mean values are reported in Table 2 and depicted on Figures 16 and 17) with the details of which groups were statistically different for each amino acid summarized in Table 4.

5.4 Discussion and Conclusions

Maternal Amino Acid Disturbances

The decreases in maternal amino acid concentrations in response to alcohol and acidemia in this study closely match findings in previous work done with a chronic exposure pattern followed by an acute alcohol or acidemic challenge (Ramadoss et al., 2008c). The decreases in some of the amino acids were more profound in the chronic followed by acute exposure than what was found in this study with a single acute challenge which suggests that compensation may not be occurring. The binge alcohol (or acidemia) exposure pattern, which mimics the typical drinking pattern of women who consume alcohol during pregnancy, consists of three consecutive days of alcohol followed by four days without alcohol and this pattern is repeated each week. Thus, it is repeated bouts rather than a continuous state. Adaptation and compensation may be easier to achieve with a chronic, continuous state, as seen with compensation in chronic disease states, versus repeated acute challenges. Acidemia and the resulting changes in

glutamine-dependent pathways may be the cause of the maternal amino acid reduction for both the alcohol and acidemia maternal groups (Ramadoss, et al., 2008c).

Also consistent with the chronic followed acute challenge paradigm in sheep by Ramadoss and colleagues (Ramadoss et al., 2008c) is the observation that glutamate significantly increased in response to alcohol. This could be a result of an inhibitory effect by alcohol on the oxidation of glutamate, thus increasing its concentration as it builds up. An increase in maternal plasma glutamate concentration was also seen in both of the alcohol plus glutamine groups and the increase was of greater magnitude than that seen in the alcohol group. The effect of alcohol plus the conversion of the increased concentration of plasma glutamine (from the acute administration of glutamine) into glutamate likely explains the higher increase in glutamate seen in the alcohol plus glutamine groups compared to the alcohol group.

While the patterns of change in amino acid concentrations in the groups receiving glutamine plus alcohol look similar to the alcohol group, different mechanisms and processes are likely occurring when glutamine is present; anabolic effects of glutamine that may explain this outcome are outlined below in the section on fetal amino acid disturbances.

Maternal blood gas changes are similar to results obtained in previous studies using this paradigm. Alcohol causes a mixed respiratory and metabolic acidosis, explaining why a greater pCO_2 elevation was required to produce a similar magnitude of acidemia as the alcohol group.

Fetal Amino Acid Disturbances

Plasma amino acid concentrations of multiple amino acids were significantly reduced in the fetus as well as the mother in response to alcohol. One exception was glutamate, which was significantly increased in the mother and increased with trend significance in the fetus. The reductions in fetal amino acids could be explained by a combination of factors (Figures 18-21), including impaired placental transfer and a change in the fetal metabolic balance between synthesis and degradation of amino acids (for example, inhibition of protein degradation would decrease plasma amino acid levels).

The acidemia group did not have the same general pattern of change fetal plasma amino acids as was seen in the alcohol group. While acidemia resulted in a reduction of maternal amino acids, an increase in the fetal plasma concentration of multiple amino acids was observed. This suggests that while acidemia and the resulting changes in glutamine-dependent pathways may be the cause of the maternal amino acid reduction for both the alcohol and acidemia maternal groups (Ramadoss, et al., 2008c), the same thing is not occurring in the fetus. Acidemia likely causes a disturbance in the balance between protein synthesis and degradation, resulting in a net increase of amino acid concentrations (Figure 20).

In the groups that received alcohol plus glutamine, there was a decrease in both maternal and fetal plasma concentrations of multiple amino acids. While the patterns of change in amino acid concentrations in the groups receiving glutamine look similar to the alcohol group, different mechanisms and processes are likely occurring when

glutamine is present (Figure 21). Glutamine is known to have trophic, anabolic effects and changes in tissue glutamine concentrations have been shown to correlate with net protein turnover; there is evidence that glutamine may both stimulate protein synthesis and inhibit protein degradation (Smith, 1990). Thus the net effect of glutamine administration could result in a decrease in plasma amino acids from anabolic stimulation rather than nutrient deprivation. This is supported by the observation that there were reductions in the plasma amino acid concentrations of many amino acids in the glutamine control group. Since plasma amino acid concentrations depend on a number of complex factors, including protein turnover, synthesis, oxidation, placental transport, and absorption, dissecting out each of these steps will require additional experiments.

Fetal Response to Acidemia

The fetal pH dropped in response to maternal alcohol administration or induction of maternal acidemia, just as a decrease in pH was seen maternally in these two groups. However, the fetal plasma amino acid changes seen in these two groups were not the same. Alcohol decreased the concentrations of multiple amino acids and acidemia increased the concentration of multiple amino acids. This increase in fetal amino acid concentrations in response to acidemia is also in direct contrast to the decrease seen in maternal plasma amino acid concentrations with acidemia. This suggests that alcohol mediates changes in amino acid concentrations in the fetus by mechanisms in addition to or independent of acidemia alone. Acidemia likely causes a disturbance in the balance between protein synthesis and degradation, resulting in a net increase of amino acid

concentrations (Figure20). Acute metabolic acidemia in fetal lambs has been shown to stimulate protein degradation (Milley, 1997).

Glutamine and pH

Acute pH changes are known to alter glutamine metabolism and acidosis has been shown to decrease plasma glutamine levels (Heitmann and Bergman, 1980; Ramadoss et al., 2008c). However, an acute administration of a single bolus of glutamine at the doses used in this study was not enough to prevent the fall in maternal or fetal arterial pH seen with alcohol administration. Since glutamine turnover is rapid, it is possible that a longer infusion period, repeating current dosages but administering the glutamine more than once, or further increasing the dosage could have a greater effect on preventing the acidemia associated with alcohol. Regardless of its ability to prevent a change in pH, glutamine may still be protective through mechanisms other than prevention of acidemia.

Glutamate Increases

Increases in maternal glutamine plasma concentrations were significantly increased in the groups that received alcohol or alcohol plus glutamine. Significance reached trend levels for the observed increase in fetal glutamine plasma concentration. Glutamate is of importance because it functions as an excitatory neurotransmitter in the brain and excessive release of glutamine in the brain can lead to excitotoxic brain injury from overexcitation of nerve cells. This is why a greater level of regulation is required for brain glutamate concentration than that observed in most other tissues. Glutamate levels are tightly regulated by uptake, glutamine synthesis, glutamate synthesis and

placental metabolism; the concentration of glutamate in brain tissue is not reflected by plasma concentrations (Smith, 2000). Under normal conditions, most free glutamine in the brain is derived from local synthesis. The blood brain barrier (BBB) helps protect the brain from changes in circulating plasma glutamate levels and the passive influx of amino acids, including glutamate, at the BBB is <1% of that observed in the blood vessels of other tissues (Smith, 2000). In addition, glutamine synthase is highly expressed in astrocytes, which allows them to uptake glutamate and convert it to glutamine, thus providing an additional mechanism for regulating brain glutamate levels (Taylor and Curthoys, 2004). Glutamate is taken up by the placenta in both the fetal sheep and human (Moores et al., 1994), which may afford protection to the fetus from high circulating levels of maternal glutamate.

There are nine know amino acid transport systems in the capillary endothelium of the BBB and their function is most important to deliver amino acids that cannot be synthesized in the brain; consequently glutamate and aspartate have much lower rates of uptake into the BBB since they can be readily synthesized in the brain. In addition, the plasma concentration for most of amino acids equals or exceeds the transport K_m (concentration at half saturation with no competition) for the corresponding transporter (Smith, 2000). The K_m for both glutamate and glutamine transport from studies in the rat brain (Smith, 2000) exceed the plasma concentration of any animals in any group in this study (either maternal or fetal) at any time point. Thus it is unlikely that the increases in either plasma glutamine or glutamate concentrations seen in this study resulted in a significant increase in brain concentration. Measurement of fetal brain

glutamate levels was not performed in this study but will be an important variable to include in future studies to confirm the absence of increased brain glutamate concentration.

Glutamine Administration

Glutamine, now considered a conditionally essential amino acid (Smith, 1990), is the most abundant amino acid in the blood and free amino acid pool in the body (Kalhan, et al., 2005). Glutamine plays an important role in the inter-organ shuttle of nitrogen and carbon and a primary oxidative fuel for enterocytes and lymphocytes (Kalhan et al., 2005). It is an important precursor for the de novo synthesis of arginine, and both glutamine and arginine have beneficial effects to the recovery of seriously ill patients; it is possible that glutamine exerts some of its actions by enhancing the availability of arginine (Ligthart-Melis, 2008; Wu and Morris, 1998). Glutamine supplementation in weanling piglets increased intestinal expression of genes necessary for cell growth and removal of oxidants, while reducing expression of genes that promote oxidative stress and immune activation which resulted in improved growth and weight gain (Wang et al., 2008). These findings demonstrate a modulatory effect on gene expression by glutamine, thus revealing a potential molecular mechanism of action for the beneficial effects of glutamine. In addition, glutamine has been shown to be a stimulus/regulator to mTOR, a pathway that balances the opposing forces of protein synthesis and degradation (Nicklin et al., 2008; Fumarola et al., 2004; Dennis, et al., 1999).

Because of its critical role in a number of physiological systems, and because there is a rapid depletion of whole body glutamine pools during acute illness, trauma,

and burns, glutamine has been studied extensively as a nutrient supplement, both parenterally and enterally (Ligthart-Melis, 2008; Smith 1990; Garlick, 2001). In low birth weight infants, parenteral glutamine supplementation reduced whole-body proteolysis and increased protein accretion (Kalhan et al., 2005). Administration of corticosteroids in humans to mimic stress conditions increased the uptake of glutamine from the splanchnic bed and could thus be a contributor to glutamine depletion under these conditions (Thibault, et al., 2008). This is noteworthy since alcohol has been demonstrated to increase both fetal and maternal glucocorticoid levels (Cudd et al., 2001b).

There is no clear recommended dietary allowance (RDA), estimated average requirement (EAR) or tolerable upper level (UL) for glutamine, but studies to date have shown no observed adverse effects for a range of clinical measurements (Garlick, 2001). Two important metabolic end products of glutamine are ammonia and glutamate, both of which are potentially neurotoxic, but in studies of glutamine safety in which neurological symptoms were assessed, no signs of adverse effects were detected (Garlick, 2001). Measurement of these intermediary metabolites will be useful in future studies assessing the efficacy of glutamine as a potential intervention strategy for FASD.

Although glutamine is stable as a dry solid, it is known to be unstable in solution, resulting in a toxic product (Furst et al., 1997); such problems can be avoided by preparing solutions freshly from the dry solid or by administering it in the form of a dipeptide (Furst, et al., 1990; Garlick, 2001). The ewes in the glutamine control and alcohol + glutamine dosed at 30 mg/kg groups received on average an approximate total

dose of 2.5 g of glutamine while the ewes in the alcohol + glutamine dosed at 100 mg/kg group received an approximate total dose of 8 g. No adverse effects of glutamine have been demonstrated when given in doses of 50-60 g/day (Garlick, 2001), so this should be well within a possible safe range of administration.

Future Studies

Results of this study support the conclusion that alcohol alters amino acid homeostasis in both the mother and the fetus. These findings may help explain the intra-uterine growth retardation and structural damage to the nervous system observed in FASD. Plasma amino acid concentrations depend on a number of complex factors, including protein turnover, synthesis, oxidation, placental transport, and absorption. Dissecting out each of these steps will require additional experiments. Glutamine has great potential as a new nutritional means to ameliorate alcohol-induced metabolic disorders and thus serve as an intervention or therapeutic strategy for FASD.

6. SUMMARY AND CONCLUSIONS

Alcohol consumption during pregnancy can result in fetal alcohol spectrum disorders (FASD) which encompass a wide range of physical, behavioral, learning, emotional and social disturbances. Many mechanisms for this array of alcohol-derived fetal injuries have been proposed, but none fully account for the deficiencies observed. Effective intervention or prevention strategies for FASD will be difficult to develop without a more complete knowledge of the mechanisms by which prenatal alcohol exposure results in neurodevelopmental damage.

This study investigated possible mechanisms for the alcohol induced neurodevelopment damage seen as a result of prenatal alcohol exposure, and also evaluated a potential intervention strategy (glutamine). These experiments all utilized the sheep model, which has distinct advantages over the rodent model for third trimester-equivalent studies (a time of increased vulnerability to the effects of alcohol).

Results from fetal hippocampal formation (pyramidal cells in the CA1 and CA2/3 fields and granule cells of the dentate gyrus) and olfactory bulb (mitral cells) stereology demonstrate a regional difference in vulnerability to alcohol in different parts of the brain at different times. It confirms the finding of other studies utilizing the rodent that show alcohol exposure during the third trimester-equivalent must occur for stereology changes to be present in the hippocampal formation. It reinforces the hippocampal formation as an important target of alcohol damage, thus directing further investigation of alterations to this brain structure.

It is known that both maternal and fetal cortisol levels increase in response to alcohol. The ability of cortisol to mediate fetal cerebellar Purkinje cell loss (known to occur with alcohol exposure) independent of alcohol was assessed with stereology. Repeated elevations in maternal cortisol did not independently produce fetal cerebellar Purkinje cell loss during the third trimester-equivalent. However, the elevations in cortisol along with other changes induced by alcohol may together mediate brain injury in FASD.

Lastly, the concentrations of circulating amino acids were measured for the first time concurrently in both the mother and fetus in response to alcohol. Alcohol caused significant reductions in both maternal and fetal circulating amino acid concentrations, which could be a cause of the growth retardation seen in FASD. The administration of a single acute dose of glutamine to the ewe, concurrent with alcohol, was not able to prevent the pH perturbations that occur in response to alcohol. It was determined that the amino acid concentrations in the fetus and mother have different patterns of alteration in response to acidemia. Glutamine was evaluated at two different doses and when administered concurrently with alcohol to the ewe as a single acute bolus, it had potential positive, anabolic effects.

These finding will help chart the course for the direction of future studies and provide a building block for continued investigations of the mechanism by which prenatal alcohol exposure mediates neurodevelopmental damage. In addition, these findings identify a novel and promising intervention strategy using glutamine; its potential warrants further investigation.

REFERENCES

Abel EL, Hannigan JH (1995) Maternal risk factors in fetal alcohol syndrome: Provocative and permissive influences. Neurotoxicol Teratol 17(4):445-62.

Abel EL, Sokol RJ (1991) A revised conservative estimate of the incidence of FAS and its economic impact. Alcohol Clin Exp Res 15(3):514-24.

Archibald SL, Fennema-Notestine C, Gamst A, Riley EP, Mattson SN, Jernigan TL (2001) Brain dysmorphology in individuals with severe prenatal alcohol exposure. Dev Med Child Neurol 43(3):148-54.

Barker DJ (1994) Maternal and fetal origins of coronary heart disease. J R Coll Physicians Lond 28(6):544-51.

Barnes DE, Walker DW (1981) Prenatal ethanol exposure permanently reduces the number of pyramidal neurons in rat hippocampus. Brain Res 227(3):333-40.

Barron S, Riley EP (1992) The effects of prenatal alcohol exposure on behavioral and neuroanatomical components of olfaction. Neurotoxicol Teratol 14(4):291-7.

Bauer-Moffett C, Altman J (1977) The effect of ethanol chronically administered to preweanling rats on cerebellar development: A morphological study. Brain Res 119(2):249-68.

Becker HC, Randall CL, Salo AL, Saulnier JL, Weathersby RT (1994) Animal research: Charting the course for FAS. Alcohol Res World 18:10-16.

Beitins IZ, Kowarski A, Shermeta DW, De Lemos RA, Migeon CJ (1970) Fetal and maternal secretion rate of cortisol in sheep: Diffusion resistance of the placenta. Pediatr Res 4(2):129-34

Bodnoff SR, Humphreys AG, Lehman JC, Diamond DM, Rose GM, Meaney MJ (1995) Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. J Neurosci 15(1 Pt 1):61-9.

Bohn MC (1984) *Glucocorticoid Induced Teratologies of the Nervous System.* Elsevier Science Publishers, New York.

Bonthius DJ, Bonthius NE, Napper RM, West JR (1992) Early postnatal alcohol exposure acutely and permanently reduces the number of granule cells and mitral cells in the rat olfactory bulb: A stereological study. J Comp Neurol 324(4):557-66.

Bonthius DJ, West JR (1990) Alcohol-induced neuronal loss in developing rats: Increased brain damage with binge exposure. Alcohol Clin Exp Res 14(1):107-18.

Bonthius DJ, West JR (1991a) Acute and long-term neuronal deficits in the rat olfactory bulb following alcohol exposure during the brain growth spurt. Neurotoxicol Teratol 13(6):611-9.

Bonthius DJ, West JR (1991b) Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. Teratology 44(2):147-63.

Caetano R, Ramisetty-Mikler S, Floyd LR, McGrath C (2006) The epidemiology of drinking among women of child-bearing age. Alcohol Clin Exp Res 30(6):1023-30.

Clarren SK, Alvord EC, Jr., Sumi SM, Streissguth AP, Smith DW (1978) Brain malformations related to prenatal exposure to ethanol. J Pediatr 92(1):64-7.

Coles C (1994) Critical periods for prenatal alcohol exposure. Alcohol Health and Research World 18:22-29.

Coulter CL, Leech RW, Schaefer GB, Scheithauer BW, Brumback RA (1993) Midline cerebral dysgenesis, dysfunction of the hypothalamic-pituitary axis, and fetal alcohol effects. Arch Neurol 50(7):771-5.

Cudd TA (2005) Animal model systems for the study of alcohol teratology. Exp Biol Med (Maywood) 230(6):389-93.

Cudd TA, Chen WJ, Parnell SE, West JR (2001a) Third trimester binge ethanol exposure results in fetal hypercapnea and acidemia but not hypoxemia in pregnant sheep. Alcohol Clin Exp Res 25(2):269-76.

Cudd TA, Chen, Wj, West JR (2001b) Fetal and maternal sheep hypothalamus pituitary adrenal axis responses to chronic binge ethanol exposure during the third trimester equivalent. Alcohol Clin Exp Res 25(7):1065-71.

Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr 15:133-59.

Day NL, Leech SL, Richardson GA, Cornelius MD, Robles N, Larkby C (2002) Prenatal alcohol exposure predicts continued deficits in offspring size at 14 years of age. Alcohol Clin Exp Res 26(10):1584-91.

Dennis PB, Fumagalli S, Thomas G (1999) Target of Rapamycin (TOR): Balancing the opposing forces of protein synthesis and degradation. Curr Opin Genetics Dev 9:49-54.

Dobbing J, Sands J (1973) Quantitative growth and development of human brain. Arch Dis Child 48(10):757-67.

Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. Early Hum Dev 3(1):79-83.

Ebrahim SH, Diekman ST, Floyd RL, Decoufle P (1999) Comparison of binge drinking among pregnant and nonpregnant women, United States, 1991-1995. Am J Obstet Gynecol 180(1 Pt 1):1-7.

Fisher SE, Atkinson M, Holzman I, David R, Van Thiel DH (1981) Effect of ethanol upon placental uptake of amino acids. Prog Biochem Pharmacol 18:216-23.

Fumarola C, La Monica S, Guidotti G (2004) Amino acid signaling through the mammalian target of rapamycin (mTOR) pathway: Role of glutamine and of cell shrinkage. J Cell Physiol 204:155-165.

Furst P, Pogan K, Stehle P (1997) Glutamine dipeptides in clinical nutrition. Nutrition 13(7-8):731-7.

Garlick PJ (2001) Assessment of the safety of glutamine and other amino acids. J Nutr 131:2556S-2561S.

Gilstrap LC, Christensen R, Clewell WH, D'Alton ME, Davidson EC, Escobedo MB (1995) Effect of corticosteroids for fetal maturation on prenatal outcomes. J Am Med Assoc 273:413-418.

Gitau R, Cameron A, Fisk NM, Glover V (1998) Fetal exposure to maternal cortisol. Lancet 352(9129)707-8.

Gladstone J, Nulman I, Koren G (1996) Reproductive risks of binge drinking during pregnancy. Reprod Toxicol 10(1):3-13.

Goodlett CR, Leo JT, O'Callaghan JP, Mahoney JC, West JR (1993) Transient cortical astrogliosis induced by alcohol exposure during the neonatal brain growth spurt in rats. Brain Res Dev Brain Res 72(1):85-97.

Hamilton DA, Kodituwakku P, Sutherland RJ, Savage DD (2003) Children with Fetal Alcohol Syndrome are impaired at place learning but not cued-navigation in a virtual Morris water task. Behav Brain Res 143(1):85-94.

Hammer RP, Jr., Scheibel AB (1981) Morphologic evidence for a delay of neuronal maturation in fetal alcohol exposure. Exp Neurol 74(2):587-96.

Hamre KM, West JR (1993) The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. Alcohol Clin Exp Res 17(3):610-22.

Heitmann RN, Bergman EN (1980) Integration of amino acid metabolism in sheep: Effects of fasting and acidosis. Am J Physiol 239(4):E248-E254.

Henderson GI, Turner D, Patwardhan RV, Lumeng L, Hoyumpa AM, Schenker S (1981) Inhibition of placental valine uptake after acute and chronic maternal ethanol consumption. J Pharacol Exp Ther 216(3):465-72.

Hennessy DP, Coghlan JP, Hardy KJ, Scoggins BA, Wintour EM (1982) The origin of cortisol in the blood of fetal sheep. J Endocrinol 95(1):71-9.

Horiguchi T, Suzuki K, Comas-Urrutia AC, Mueller-Heubach E, Boyer-Milic AM, Baratz RA, Morishima HO, James LS, Adamsons K (1971) Effect of ethanol upon uterine activity and fetal acid-base state of the rhesus monkey. Am J Obstet Gynecol 109(6):910-7.

Jacobson SW, Bihun JT, Chiodo LM (1999) Effects of prenatal alcohol and cocaine exposure on infant cortisol levels. Dev Psychopathol 11(2):195-208.

Jameson RR, Seidler FJ, Qiao D, Slotkin TA (2006) Adverse neurodevelopmental effects of dexamethasone modeled in PC12 cells: Identifying the critical stages and concentration thresholds for the targeting of cell acquisition, differentiation and viability. Neuropsychopharmacology 31(8):1647-58.

Jones CT, Luther E, Richie JWK, Worthington D (1975) The clearance of ACTH from the plasma of adult and fetal sheep. Endocrinology 96:231-234.

Jones KL, Smith DW, Ulleland CN, Streissguth P (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1(7815):1267-71.

Kalhan SC, Parimi PS, Gruca LL, Hanson RW (2005) Glutamine supplement with parenteral nutrition decreases whole body proteolysis in low birth weight infants. J Pediatr 146:642-647.

Karl PI, Kwun R, Slonim A, Fisher SE (1995) Ethanol elevates fetal serum glutamate levels in the rat. Alcohol Clin Exp Res 19(1):177-81.

Kim CK, Giberson PK, Yu W, Zoeller RT, Weinberg J (1999) Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal responses to chronic cold stress in rats. Alcohol Clin Exp Res 23(2):301-10.

Kirstein CL, Philpot RM, Dark T (1997) Fetal alcohol syndrome: Early olfactory learning as a model system to study neurobehavioral deficits. Int J Neurosci 89(1-2):119-32.

Kuhn KS, Schuhmann K, Stehle P, Darmaun D, Furst P (1999) Determination of glutamine in muscle protein facilitates accurate assessment of proteolysis and de novo synthesis-derived endogenous glutamine production. Am J Clin Nutr 70(4):484-9.

Kwon H, Spencer TE, Bazer FW, Wu G (2003) Developmental changes of amino acids in ovine fetal fluids. Biol Reprod 68(5):1813-20.

Lamminpaa A, Vilska J (1991) Acid-base balance in alcohol users seen in an emergency room. Vet Hum Toxicol 33(5):482-5.

Lemoine P, Harousseau H, Borteyru JP, M JC (1968) Les enfants de parents alcooliques: Anomalies observees, a propos de 127 cas. Ouest Med 21:476-482.

Ligthart-Melis GC, van de Poll MCG, Boelens PG, Dejong CHC, Deutz NEP, van Leeuwen PAM (2008) Glutamine is an important precursor for de novo synthesis of arginine in humans. Am J Clin Nutr 87:1282-9.

Lin GW (1981) Effect of ethanol feeding during pregnancy on placental transfer of alpha-aminoisobutyric acid in the rat. Life Sci 28(6):595-601.

Livy DJ, Miller EK, Maier SE, West JR (2003) Fetal alcohol exposure and temporal vulnerability: Effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicol Teratol 25(4):447-58.

Lupton C, Burd L, Harwood R (2004) Cost of fetal alcohol spectrum disorders. Am J Med Genet C Semin Med Genet 127C(1):42-50.

Maier SE, Miller JA, Blackwell JM, West JR (1999) Fetal alcohol exposure and temporal vulnerability: Regional differences in cell loss as a function of the timing of binge-like alcohol exposure during brain development. Alcohol Clin Exp Res 23(4):726-34.

Maier SE, West JR (2001a) Drinking patterns and alcohol-related birth defects. Alcohol Res Health 25(3):168-74.

Maier SE, West JR (2001b) Regional differences in cell loss associated with binge-like alcohol exposure during the first two trimesters equivalent in the rat. Alcohol 23(1):49-57.

Marquis SM, Leichter J, Lee M (1984) Plasma amino acids and glucose levels in the rat fetus and dam after chronic maternal alcohol consumption. Biol Neonate 46(1):36-43.

Mastorakos G, Ilias I (2003) Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Ann N Y Acad Sci 997:236-49.

Mates JM, Perez-Gomez C, Nunez de Castro I, Asenjo M, Marquez J (2002) Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. Int J Biochem Cell Biol 34(5):439-58.

Matthews SG (2000) Antenatal glucocorticoids and programming of the developing CNS. Pediatr Res 47(3):291-300.

Mattson SN, Jernigan TL, Riley EP (1994) MRI and prenatal alcohol exposure: Images provide insight into FAS. Alcohol Health and Research World 18:49-52.

Mattson SN, Riley EP, Jernigan TL, Ehlers CL, Delis DC, Jones KL, Stern C, Johnson KA, Hesselink JR, Bellugi U (1992) Fetal alcohol syndrome: A case report of neuropsychological, MRI and EEG assessment of two children. Alcohol Clin Exp Res 16(5):1001-3.

Mattson SN, Schoenfeld AM, Riley EP (2001) Teratogenic effects of alcohol on brain and behavior. Alcohol Res Health 25(3):185-91.

May PA, Gossage JP (2001) Estimating the prevalence of fetal alcohol syndrome. A summary. Alcohol Res Health 25(3):159-67.

McGowan JE, Sysyn G, Peterson KH, Sadowska GB, Mishra OP, Delivoria-Papadopoulos M, Stonestreet BS (2000) Effect of dexamethasone treatment on maturational changes in the NMDA receptor in sheep brain. J Neurosci 20(19):7424-9.

Miller MW (1986) Effects of alcohol on the generation and migration of cerebral cortical neurons. Science 233(4770):1308-11.

Miller MW (1995) Generation of neurons in the rat dentate gyrus and hippocampus: Effects of prenatal and postnatal treatment with ethanol. Alcohol Clin Exp Res 19(6):1500-9.

Miller MW, Nowakowski RS (1991) Effect of prenatal exposure to ethanol on the cell cycle kinetics and growth fraction in the proliferative zones of fetal rat cerebral cortex. Alcohol Clin Exp Res 15(2):229-32.

Miller MW, Robertson S (1993) Prenatal exposure to ethanol alters the postnatal development and transformation of radial glia to astrocytes in the cortex. J Comp Neurol 337(2):253-66.

Milley JR (1997) Ovine fetal leucine kinetics and protein metabolism during acute metabolic acidosis. Am J Physiol 272(2 Pt 1):E275-81.

Moores RR, Jr., Vaughn PR, Battaglia FC, Fennessey PV, Wilkening RB, Meschia G (1994) Glutamate metabolism in fetus and placenta of late-gestation sheep. Am J Physiol 267(1 Pt 2):R89-96.

Murphy BP, Inder TE, Huppi PS, Warfield S, Zientara GP, Kikinis R, Jolesz FA, Volpe JJ (2001) Impaired cerebral cortical gray matter growth after treatment with dexamethasone for neonatal chronic lung disease. Pediatrics 107(2):217-21.

Murphy VE, Smith R, Giles WB, Clifton VL (2006) Endocrine regulation of human fetal growth: The role of the mother, placenta, and fetus. Endocr Rev 27(2):141-69.

Neu J (2001) Glutamine in the fetus and critically ill low birth weight neonate: Metabolism and mechanism of action. J Nutr 131(9 Suppl):2585S-9S; discussion 2590S.

Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, Murphy LO (2009) Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 136:521-534.

Nissim I (1999) Newer aspects of glutamine/glutamate metabolism: The role of acute pH changes. Am J Physiol 277(4 Pt 2):F493-7.

Obernier JA, White AM, Swartzwelder HS, Crews FT (2002) Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. Pharmacol Biochem Behav 72(3):521-32.

Padmanabhan R, Ibrahim A, Bener A (2002) Effect of maternal methionine pre-treatment on alcohol-induced exencephaly and axial skeletal dysmorphogenesis in mouse fetuses. Drug Alcohol Depend 65(3):263-81.

Peiffer J, Majewski F, Fischbach H, Bierich JR, Volk B (1979) Alcohol embryo- and fetopathy. Neuropathology of 3 children and 3 fetuses. J Neurol Sci 41(2):125-37.

Penton Z (1985) Headspace measurement of ethanol in blood by gas chromatography with a modified autosampler. Clin Chem 31(3):439-41.

Ramadoss J, Hogan HA, Given JC, West JR, Cudd TA (2006) Binge alcohol exposure during all three trimesters alters bone strength and growth in fetal sheep. Alcohol 38:185-92.

Ramadoss J, Lunde ER, Chen WJ, West JR, Cudd TA (2007a) Temporal vulnerability of fetal cerebellar Purkinje cells to chronic binge alcohol exposure: Ovine model. Alcohol Clin Exp Res 31(10):1738-45.

Ramadoss J, Lunde ER, Pina KB, Chen WJ, Cudd TA (2007b) All three trimester binge alcohol exposure causes fetal cerebellar purkinje cell loss in the presence of maternal hypercapnea, acidemia, and normoxemia: Ovine model. Alcohol Clin Exp Res 31(7):1252-8.

Ramadoss J, Tress U, Chen WJ, Cudd TA (2008b) Maternal adrenocorticotropin, cortisol, and thyroid hormone responses to all three-trimester equivalent repeated binge alcohol exposure: Ovine model. Alcohol 42(3):199-205.

Ramadoss J, Wu G, Cudd TA (2008c) Chronic binge ethanol mediated acidemia reduces availability of glutamine and related amino acids in maternal plasma of pregnant sheep. Alcohol (42): 657-666.

Riikonen R, Salonen I, Partanen K, Verho S (1999) Brain perfusion SPECT and MRI in foetal alcohol syndrome. Dev Med Child Neurol 41(10):652-9.

Riley EP, McGee CL (2005) Fetal alcohol spectrum disorders: An overview with emphasis on changes in brain and behavior. Exp Biol Med (Maywood) 230(6):357-65.

Riley EP, McGee CL, Sowell ER (2004) Teratogenic effects of alcohol: A decade of brain imaging. Am J Med Genet C Semin Med Genet 127C(1):35-41.

Rosett HL, Weiner L (1983) *Alcohol and the Fetus*. Oxford University Press, New York.

Sahn SA, Lakshminarayan S, Pierson DJ, Weil JV (1975) Effect of ethanol on the ventilator responses to oxygen and carbon dioxide in the man. Clin Sci Mol Med 49(1):33-8.

Schenker S, Becker HC, Randall CL, Phillips DK, Baskin GS, Henderson GI (1990) Fetal alcohol syndrome: Current status of pathogenesis. Alcohol Clin Exp Res 14(5):635-47.

Shetty AK, Burrows RC, Wall KA, Phillips DE (1994) Combined pre- and postnatal ethanol exposure alters the development of Bergmann glia in rat cerebellum. Int J Dev Neurosci 12(7):641-9.

Smith DE, Foundas A, Canale J (1986) Effect of perinatally administered ethanol on the development of the cerebellar granule cell. Exp Neurol 92(3):491-501.

Smith QR (2000) Transport of glutamate and other amino acids at the blood-brain barrier. J Nutr 130(4S Suppl):1016S-22S.

Smith RJ, Wilmore DW (1990) Glutamine nutrition and requirements. Journal of Parenteral and Enteral Nutrition 14:945-995.

Sokol RJ, Clarren SK (1989) Guidelines for use of terminology describing the impact of prenatal alcohol on the offspring. Alcohol Clin Exp Res 13(4):597-8.

Sokol RJ, Delaney-Black V, Nordstrom B (2003) Fetal alcohol spectrum disorder. JAMA 290(22):2996-9.

Sowell ER, Jernigan TL, Mattson SN, Riley EP, Sobel DF, Jones KL (1996) Abnormal development of the cerebellar vermis in children prenatally exposed to alcohol: Size reduction in lobules I-V. Alcohol Clin Exp Res 20(1):31-4.

Sowell ER, Mattson SN, Thompson PM, Jernigan TL, Riley EP, Toga AW (2001) Mapping callosal morphology and cognitive correlates: Effects of heavy prenatal alcohol exposure. Neurology 57(2):235-44.

Stratton K, Howe C, Battaglia F (1996) *Fetal Alcohol Syndrome. Diagnosis, Epidemiology, Prevention and Treatment.* Institute of Medicine. National Academy Press, Washington, DC.

Sulik K, Lauder J, Dehart D (1984) Brain malformations in prenatal mice following acute maternal ethanol administration. Int J Develop Neurosci 2:203-214.

Taylor L, Curthoys NP (2004) Glutamine metabolism. Biochem Mol Biol Edu 32(5):291-304.

Thibault R, Welch S, Mauras N, Sager B, Altomare A, Haymond M, Darmaun D (2008) Corticosteroids increase glutamine utilization in human splanchnic bed. Am J Physiol Gastrointest Liver Physiol 294:G548-G553.

Uecker A, Nadel L (1996) Spatial locations gone awry: Object and spatial memory deficits in children with fetal alcohol syndrome. Neuropsychologia 34(3):209-23.

Urso T, Cavaler JS, VanThiel DH (1981) Blood ethanol levels in sober alcoholic users seen in an emergency room. Life Sci 28:1053-1056.

Vaughn PR, Lobo C, Battaglia FC, Fennessey PV, Wilkening RB, Meschia G (1995) Glutamine-glutamate exchange between placenta and fetal liver. Am J Physiol 268(4 Pt 1):E705-11.

Volk B, Maletz J, Tiedemann M, Mall G, Klein C, Berlet HH (1981) Impaired maturation of Purkinje cells in the fetal alcohol syndrome of the rat. Light and electron microscopic investigations. Acta Neuropathol 54(1):19-29.

Wang J, Chen L, Li P, Li X, Zhou H, Wang F, Li D, Yin Y, Wu G (2008) Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. J Nutr 138:1025-1032.

Weichsler ME Jr (1977) The therapeutic use of glucocorticoid hormones in the perinatal period: Potential neurologic hazards. Ann Neurol 2:364-366.

Weinberg J (1989) Prenatal ethanol exposure alters adrenocortical development of offspring. Alcohol Clin Exp Res 13(1):73-83.

Weinberg J, Bezio S (1987) Alcohol-induced changes in pituitary-adrenal activity during pregnancy. Alcohol Clin Exp Res 11(3):274-80.

West JR (1987) Fetal alcohol-induced brain damage and the problem of determining temporal vulnerability: A review. Alcohol Drug Res 7(5-6):423-41.

West JR, Chen WJ, Pantazis NJ (1994) Fetal alcohol syndrome: The vulnerability of the developing brain and possible mechanisms of damage. Metab Brain Dis 9(4):291-322.

West JR, Hamre KM, Cassell MD (1986) Effects of ethanol exposure during the third trimester equivalent on neuron number in rat hippocampus and dentate gyrus. Alcohol Clin Exp Res 10(2):190-7.

West JR, Parnell SE, Chen WJ, Cudd TA (2001) Alcohol-mediated Purkinje cell loss in the absence of hypoxemia during the third trimester in an ovine model system. Alcohol Clin Exp Res 25(7):1051-7.

Wisniewski K, Dambska M, Sher JH, Qazi Q (1983) A clinical neuropathological study of the fetal alcohol syndrome. Neuropediatrics 14(4):197-201.

Wood CE, Chen HG (1989) Acidemia stimulates ACTH, vasopressin, and heart rate responses in fetal sheep. Am J Physiol 257(2 Pt 2):R344-9.

Wu, G (2009) Amino acids: Metabolism, functions, and nutrition. Amino Acids 37:1-17.

Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE (2004) Maternal nutrition and fetal development. J Nutr 134(9):2169-72.

Wu G, Davis PK, Flynn NE, Knabe DA, Davidson JT (1997) Endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in postweaning growing pigs. J Nutr 127(12):2342-9.

Wu G, Morris SM, Jr. (1998) Arginine metabolism: Nitric oxide and beyond. Biochem J 336 (Pt 1):1-17.

Wu G, Pond WG, Ott T, Bazer FW (1998) Maternal dietary protein deficiency decreases amino acid concentrations in fetal plasma and allantoic fluids of pigs. J Nutr 128(5):894-902.

Zehtabchi S, Sinert R, Baron BJ, Paladino L, Yadak K (2005) Does ethanol explain the acidosis commonly seen in ethanol-intoxicated patients? Clin Toxicol (Phila) 43(3):161-6.

APPENDIX A

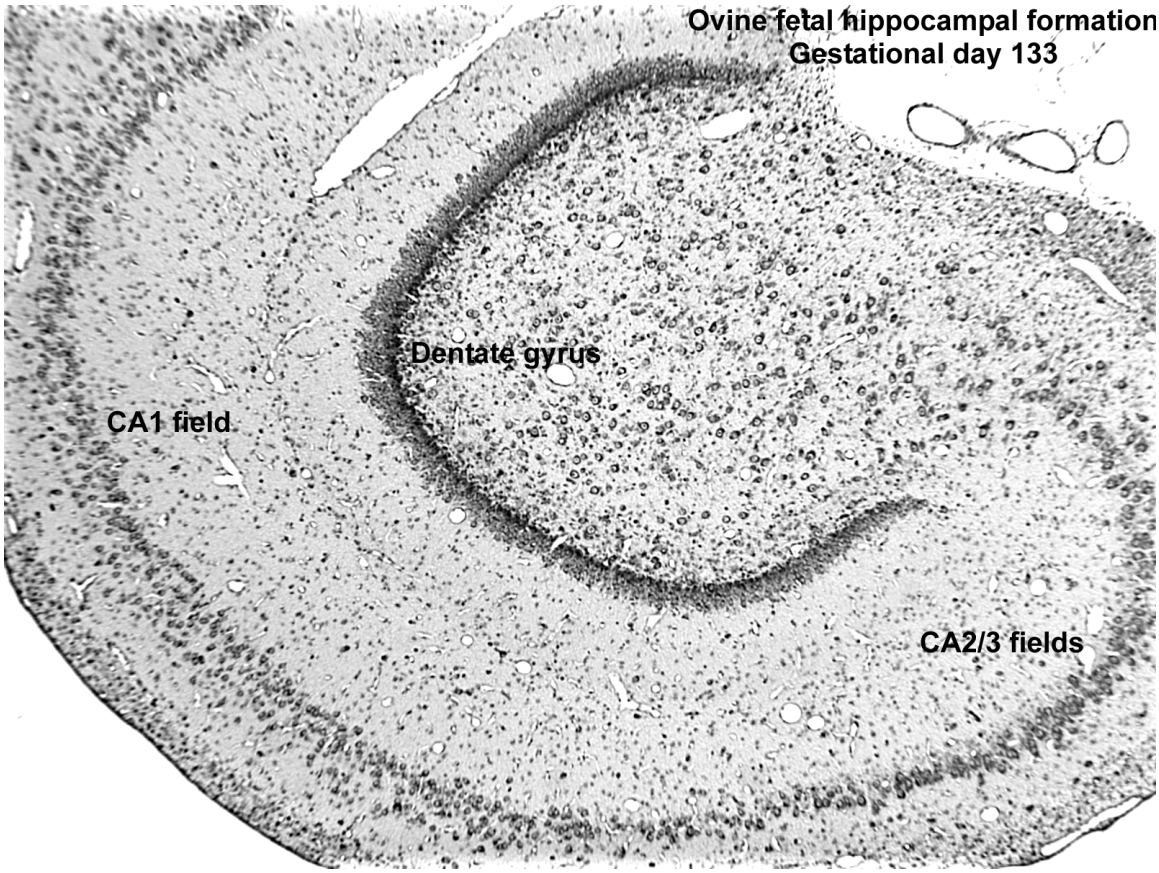

Figure 1: Representative coronal section of the ovine fetal hippocampal formation from the control group. The section illustrates well formed dentate gyrus, CA1, and CA2/3 pyramidal fields by gestational day 133 (term = 147).

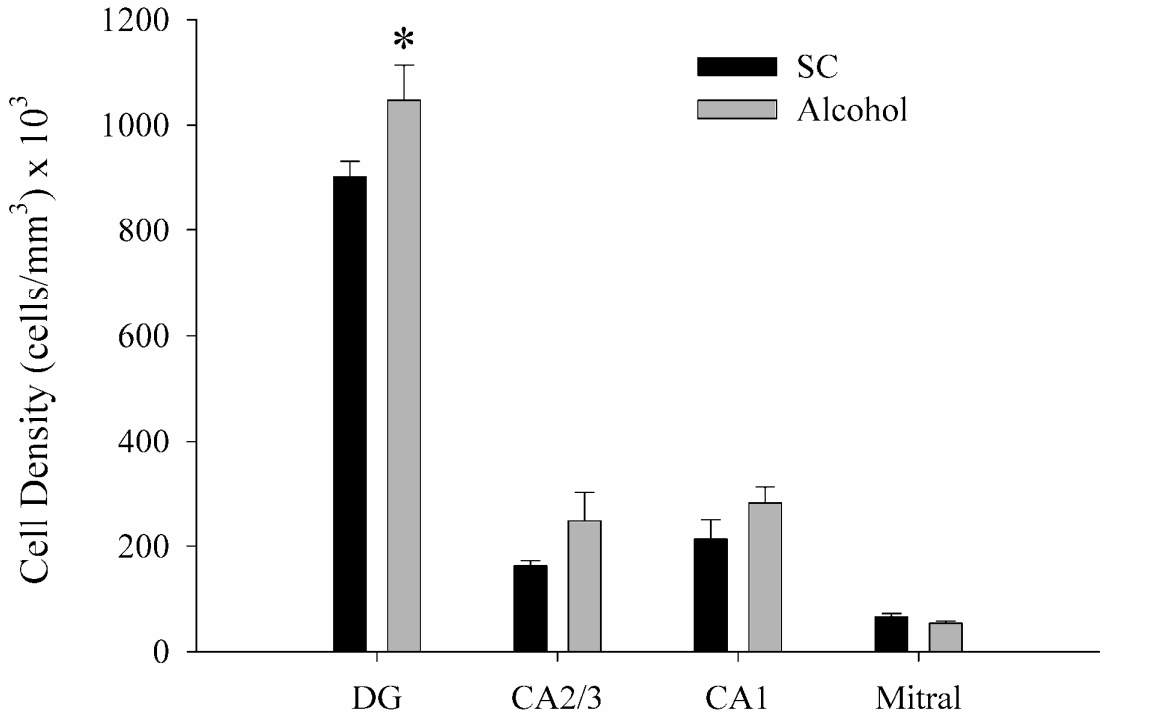

Figure 2: No differences could be detected in the total cell number between the control and alcohol groups. The density of the dentate gyrus granule cells (DG) trended higher (*) in response to all three trimester binge alcohol exposure *in utero*. No such differences were found for the pyramidal cells of the CA2/3 field, the CA1 field, and the mitral cells of the olfactory bulb. Values are mean ± SEM.

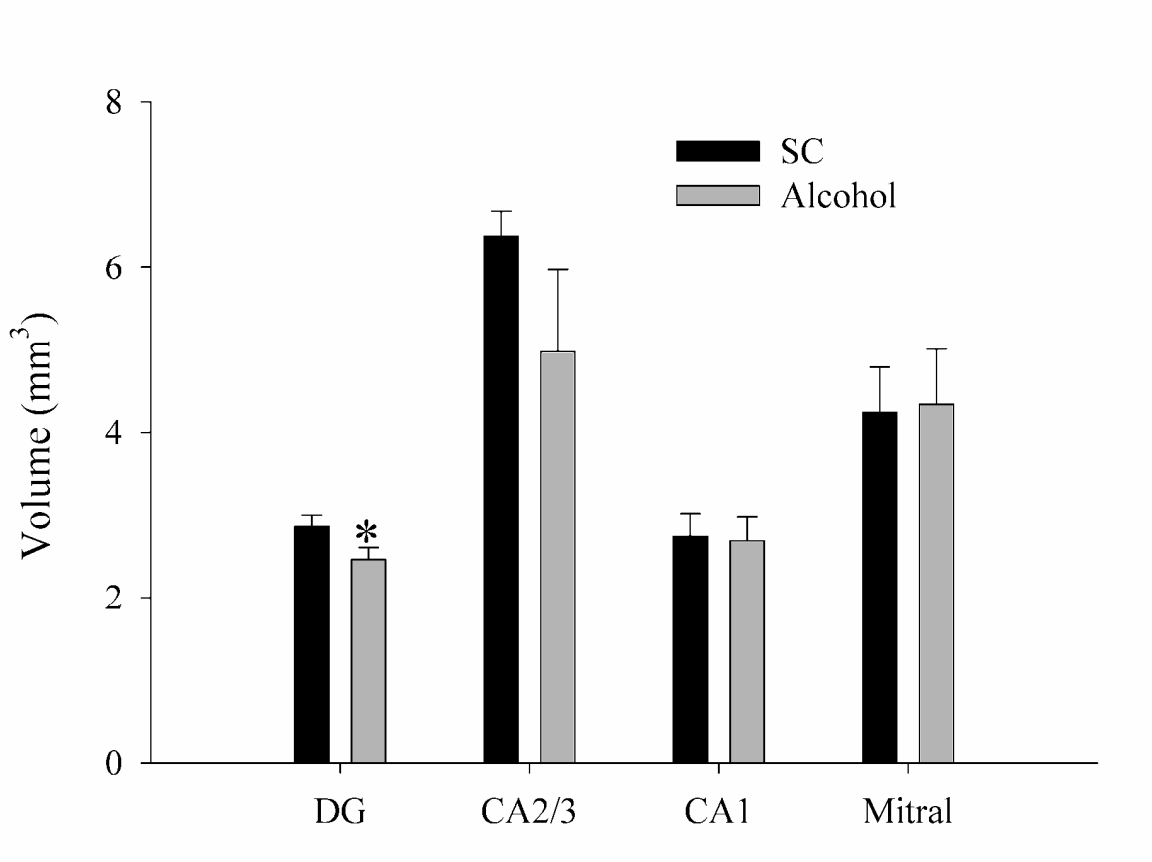

Figure 3: The volume of the dentate gyrus (DG) trended to be lower (*) in the all three trimester binge alcohol group. The reference volume for the CA2/3 field, CA1 field and the mitral cells were not different between groups. Values are mean $\pm$ SEM.

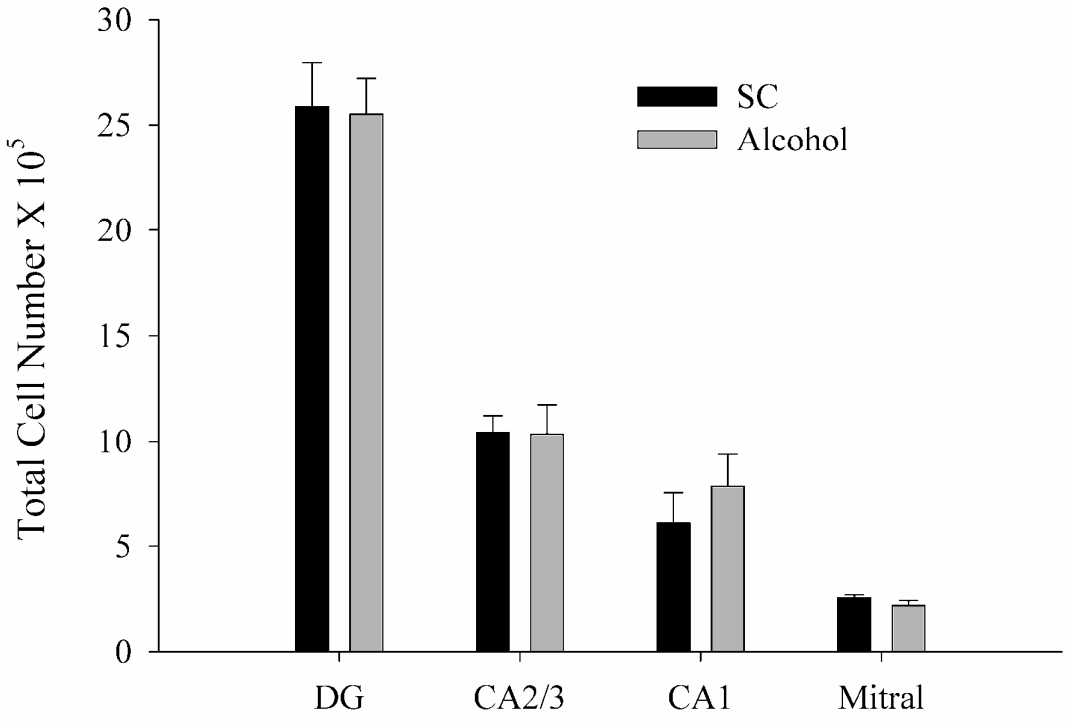

Figure 4: The estimated mean total number of fetal dentate gyrus granule cells (DG), CA2/3 pyramidal cells, CA1 pyramidal cells, and the mitral cells was not different between control and alcohol groups. Values are mean $\pm$ SEM.

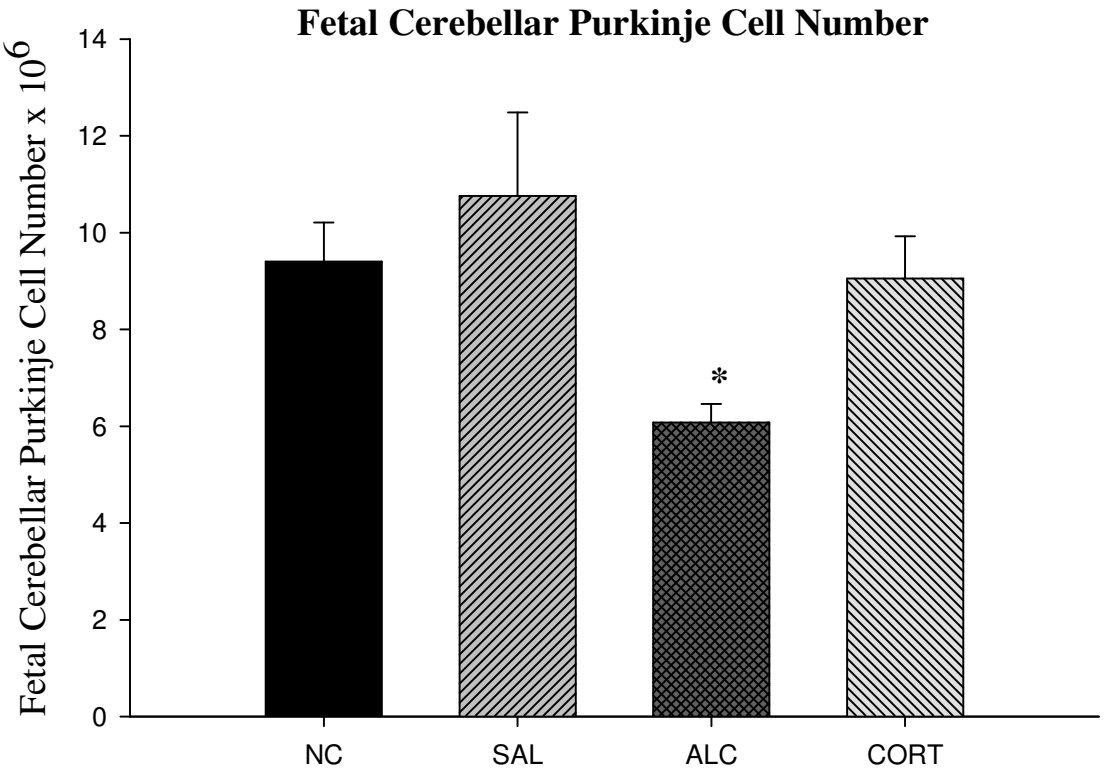

Figure 5: Estimated fetal cerebellar Purkinje cell number (x 10^6). Alcohol exposure significantly decreased total Purkinje cell number compared to the normal control, saline control, and the cortisol treatment groups. No differences were noted between the controls and the cortisol group.

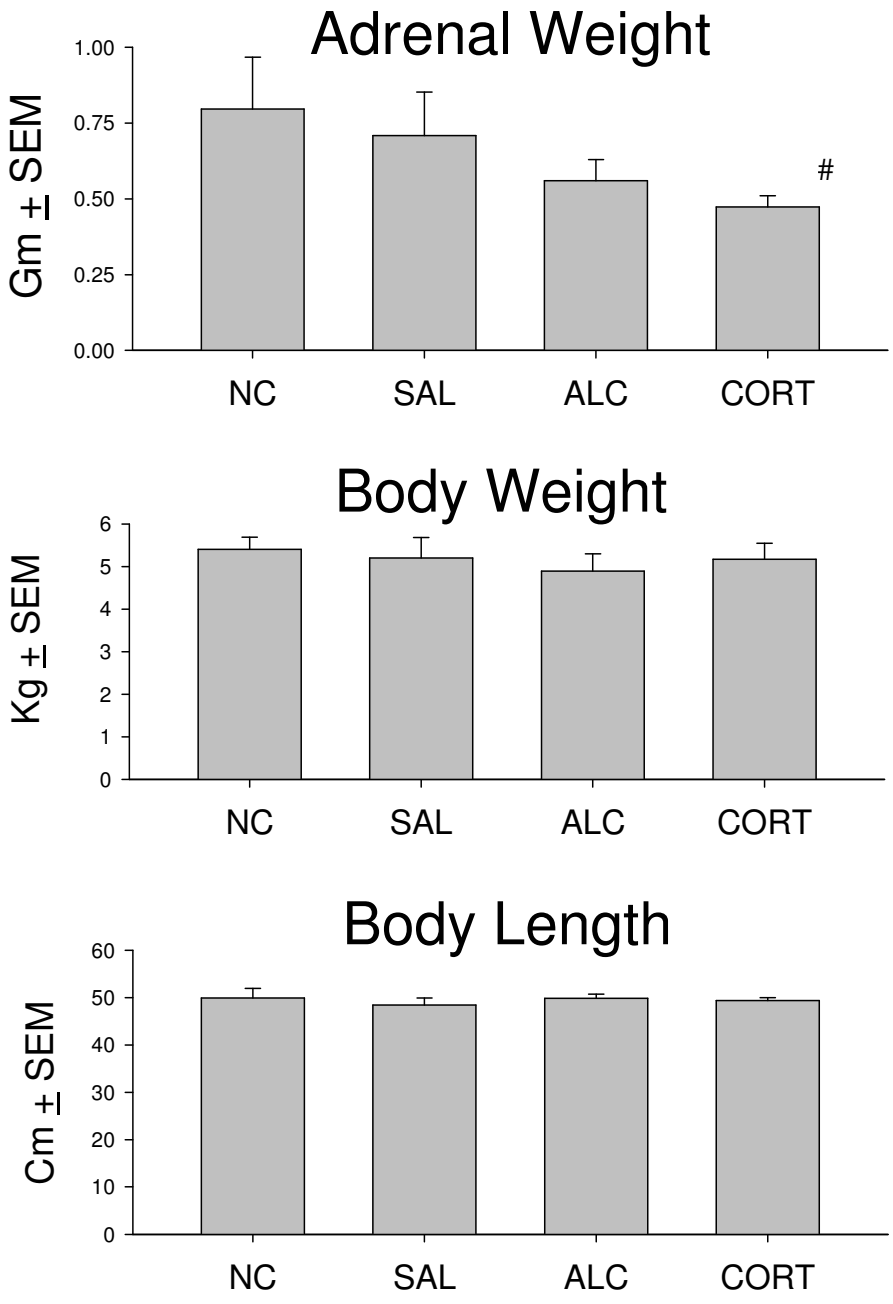

Figure 6: Fetal growth measures. Fetal whole body weight and body length (crown to rump) were not different among groups. Fetal adrenal weight had a lower trend in the cortisol group.

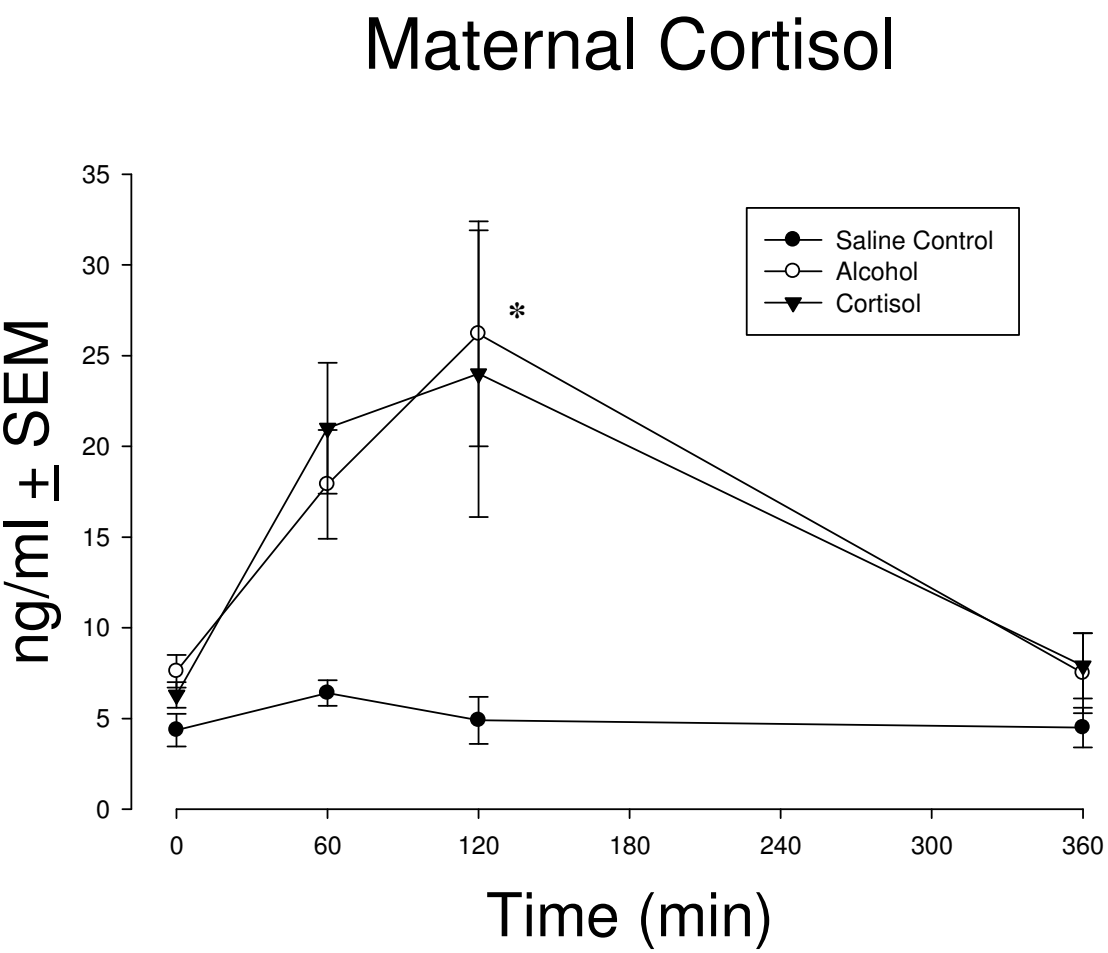

Figure 7: Maternal plasma cortisol levels (from experiment on gestational day 132). The magnitude of elevation in cortisol in response to alcohol was mimicked in the cortisol group by infusing pregnant ewes with hydrocortisone for 6 hours on each day of the experiment. The cortisol and alcohol groups were not significantly different at any time point, but were significantly increased at 120 minutes compared to the saline control group. Maternal cortisol levels were not significantly altered in the saline control group at any time point.

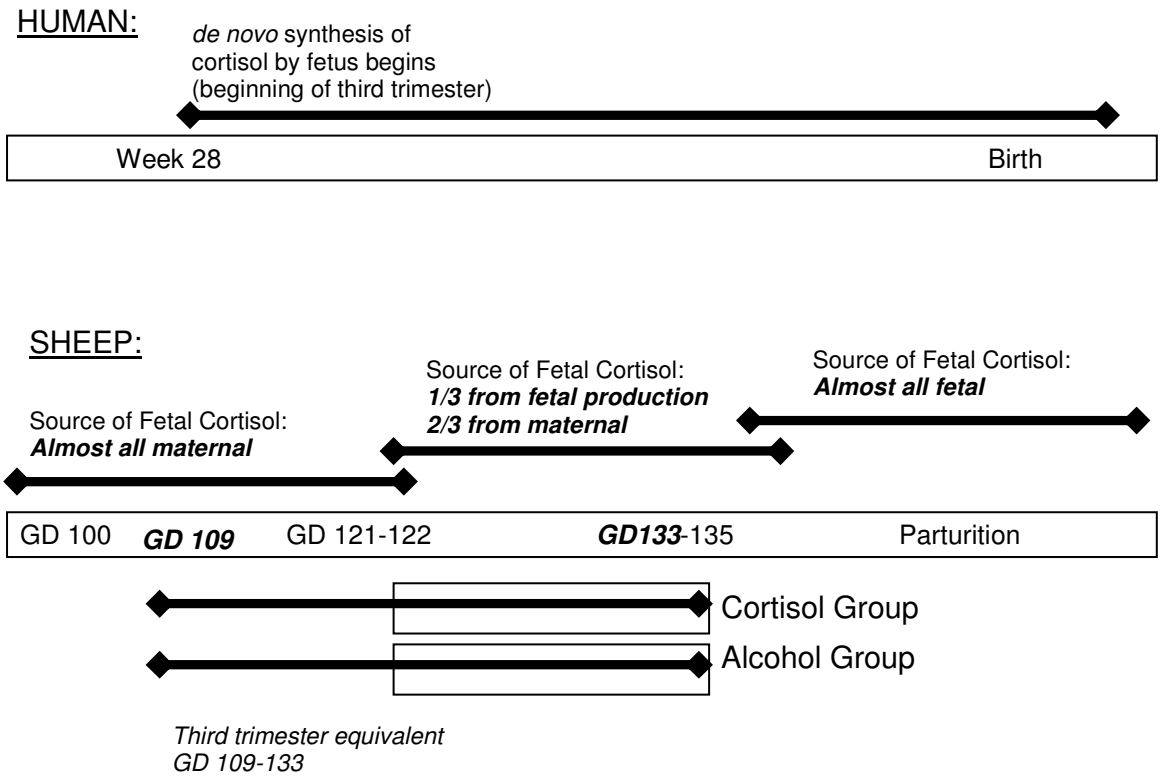

Figure 8: Timeline of fetal cortisol steroid genesis in the sheep and human

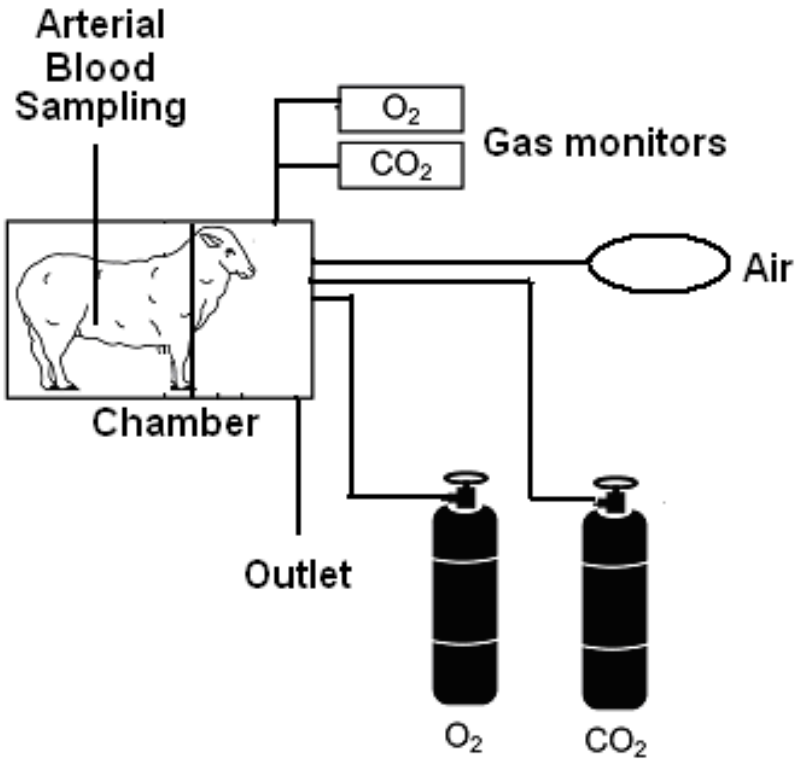

Figure 9: Illustration of the ventilation chamber. Maternal blood gases in the acidemic group were manipulated by placing the ewe in a chamber and manipulating the inspired gases to mimic the change in maternal arterial pH produced by alcohol. The front half of the subject was confined inside the plexiglass chamber, while the rear half was accessible to the investigator for sampling blood. The fractional concentrations of oxygen and carbon dioxide in the chamber were measured using gas monitors.

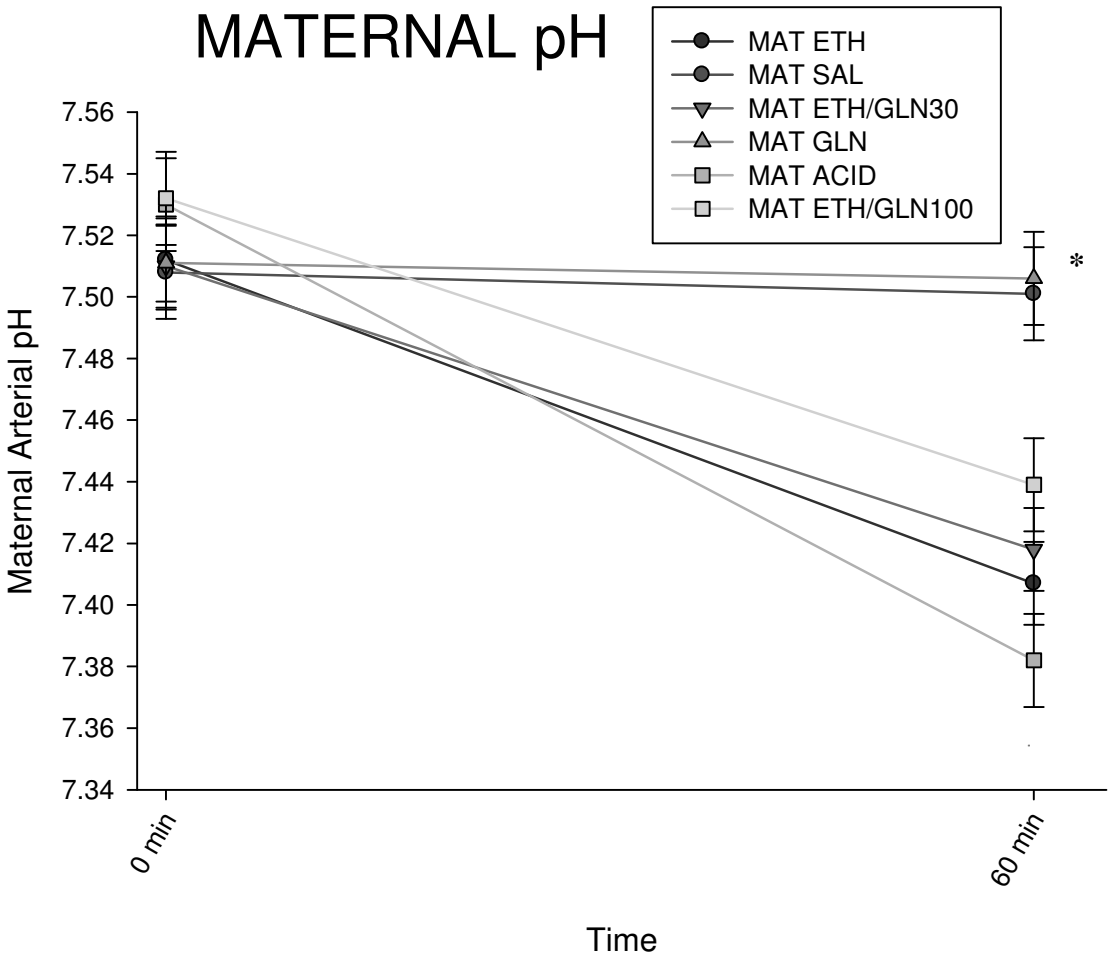

Figure 10: Maternal arterial pH was significantly higher in the saline and glutamine control groups compared to all other groups at 60 minutes, but they did not differ from each other. There was not a statistical difference among the acidemia, alcohol, or alcohol plus glutamine groups. There was no difference among any of the groups at 0 minutes.

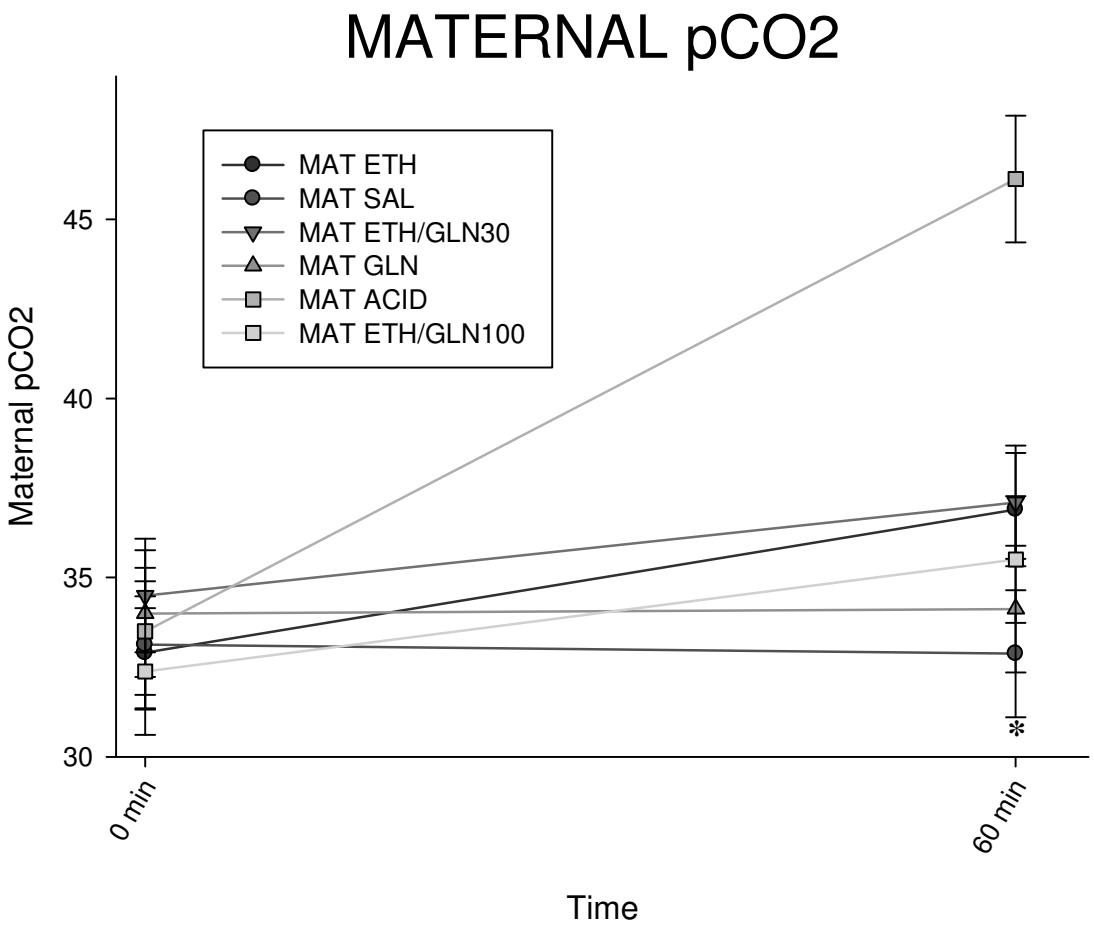

Figure 11: Maternal CO_2 was significantly higher in the acidemia group compared to all other groups at 60 minutes; the other groups did not differ from each other. There was no difference among any of the groups at 0 minutes.

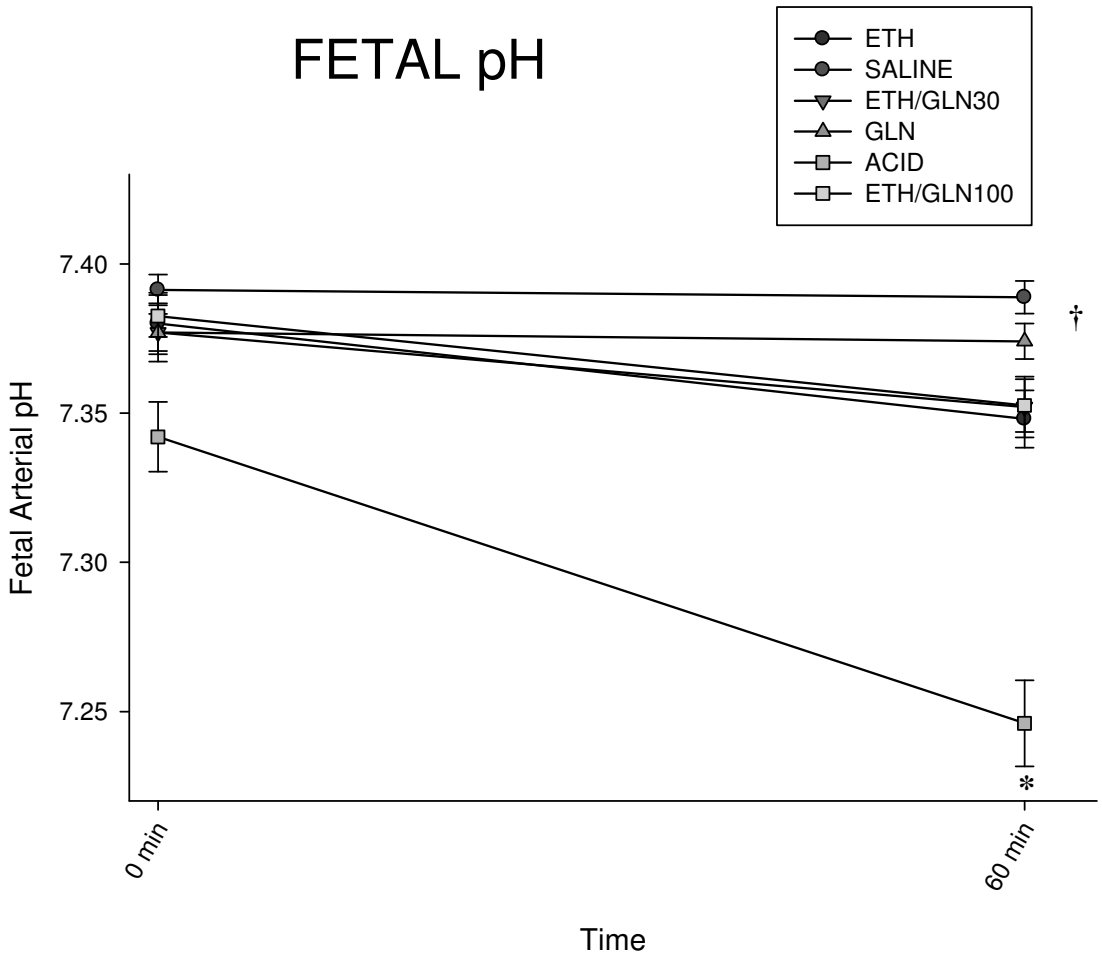

Figure 12: *Fetal arterial pH was significantly lower at 60 minutes in the acidemia group compared to all other groups. †The alcohol group had a significantly lower pH compared to the saline and glutamine control groups at 60 minutes, but did not differ from the alcohol plus glutamine groups. There was no difference among groups at 0 minutes.

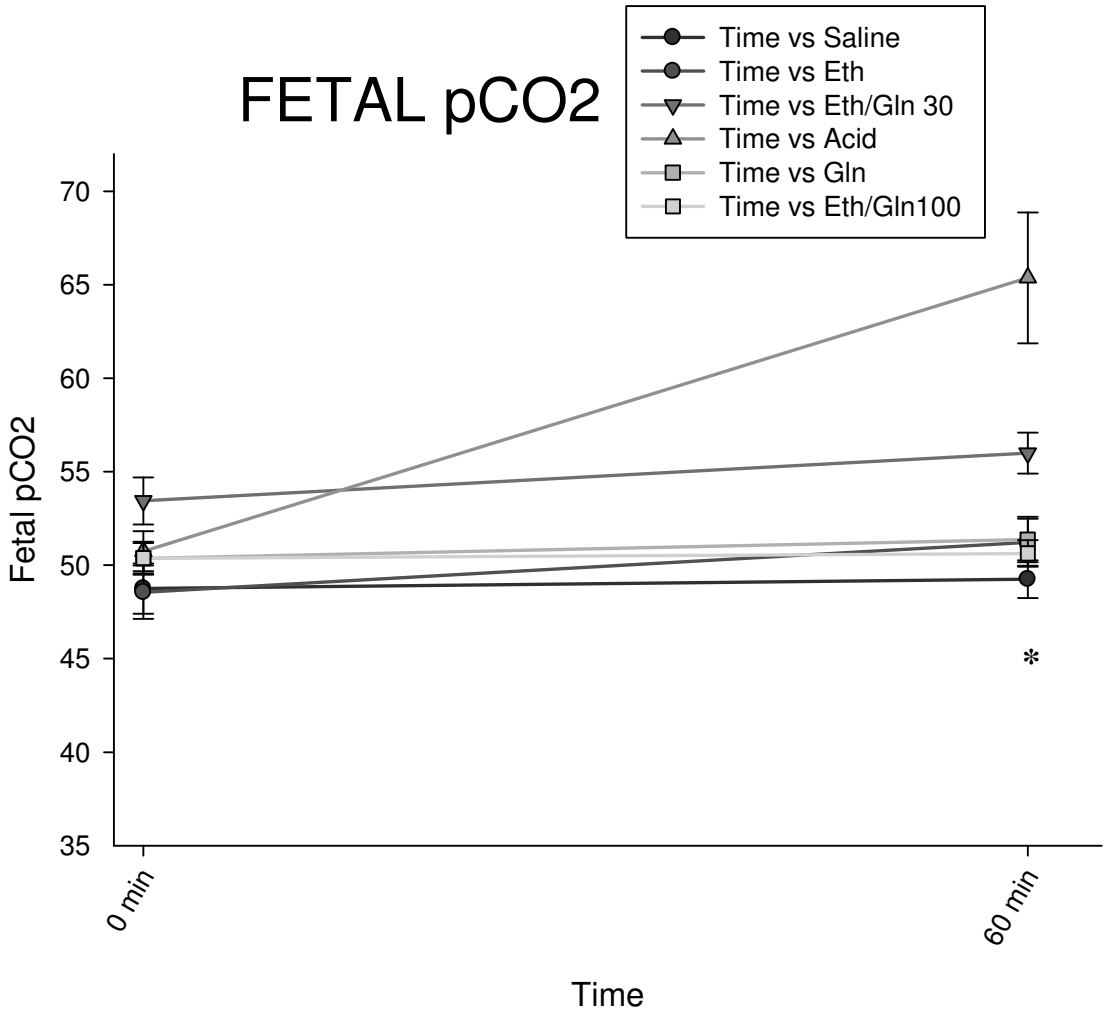

Figure 13: Fetal pCO2 was significantly increased in the acidemia at 60 minutes compared to all other groups; there was no difference among the other groups at 60 minutes. There was no difference among groups at 0 minutes.

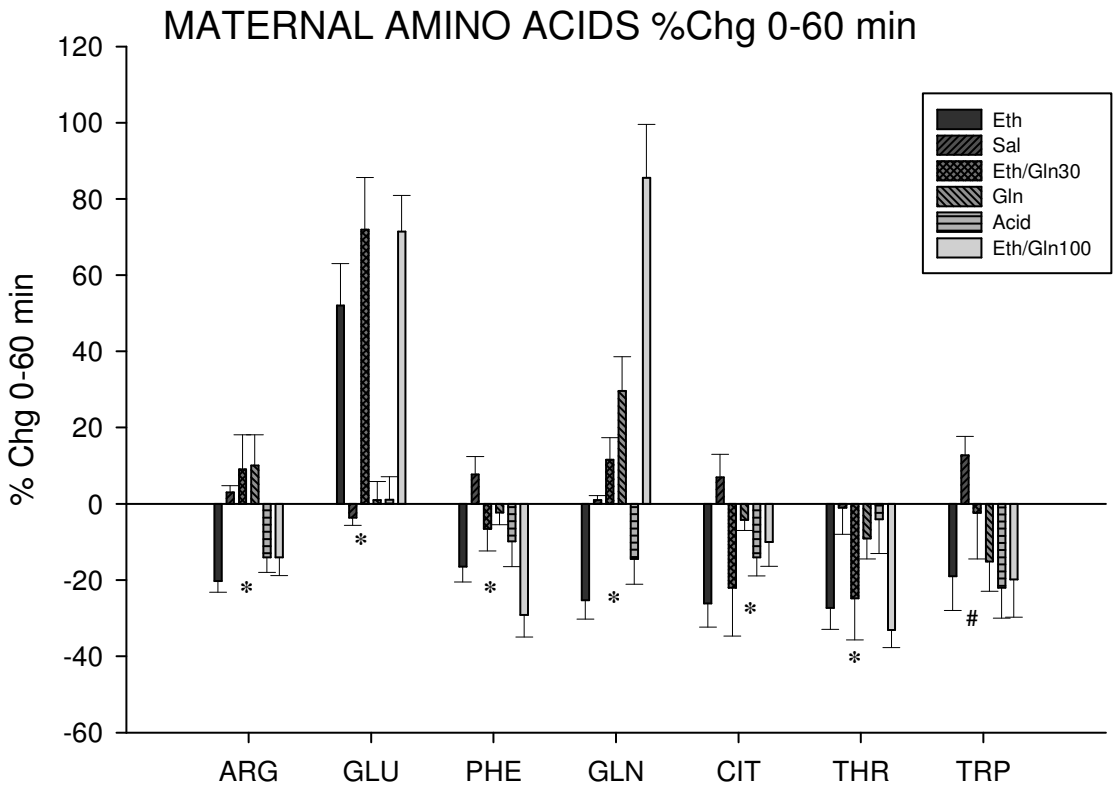

Figure 14: Maternal plasma concentrations of amino acids represented as percent change from 0 to 60 minutes. Significance (*) and trend (#) are indicated on the graph with details on which groups differed in Table 3.

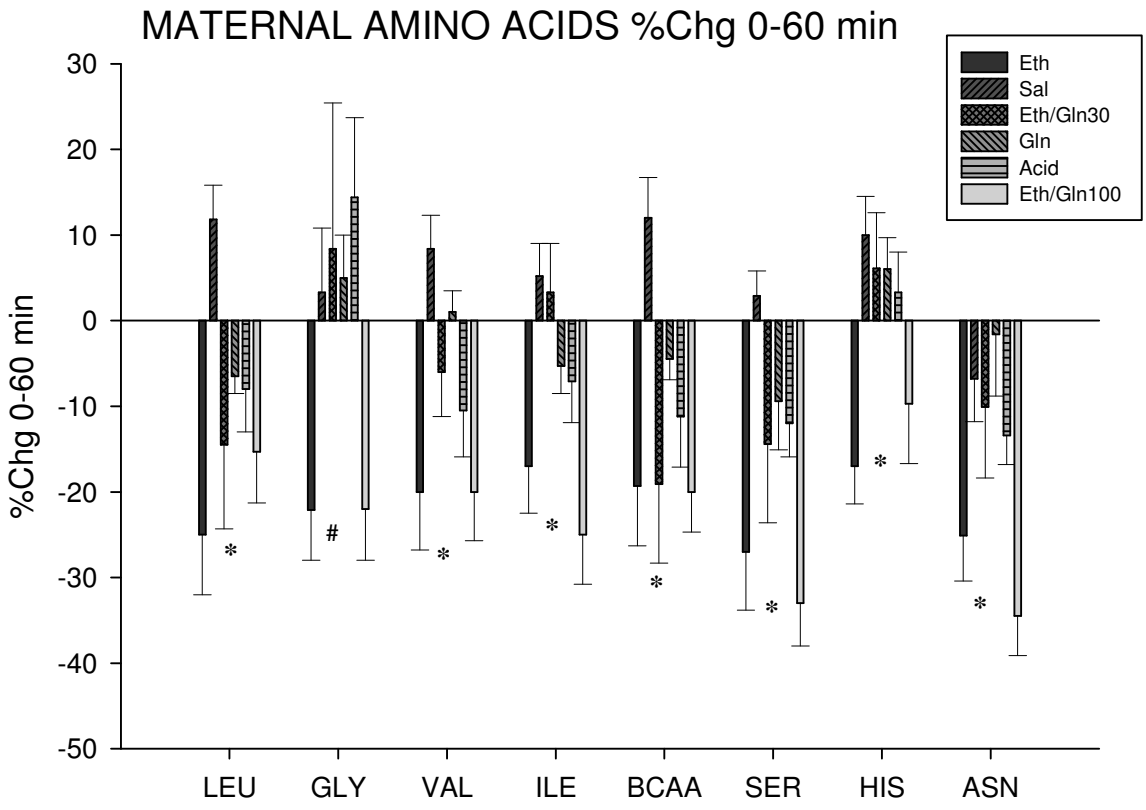

Figure 15: Maternal plasma concentrations of amino acids represented as percent change from 0 to 60 minutes. Significance (*) and trend (#) are indicated on the graph with details on which groups differed in Table 3.

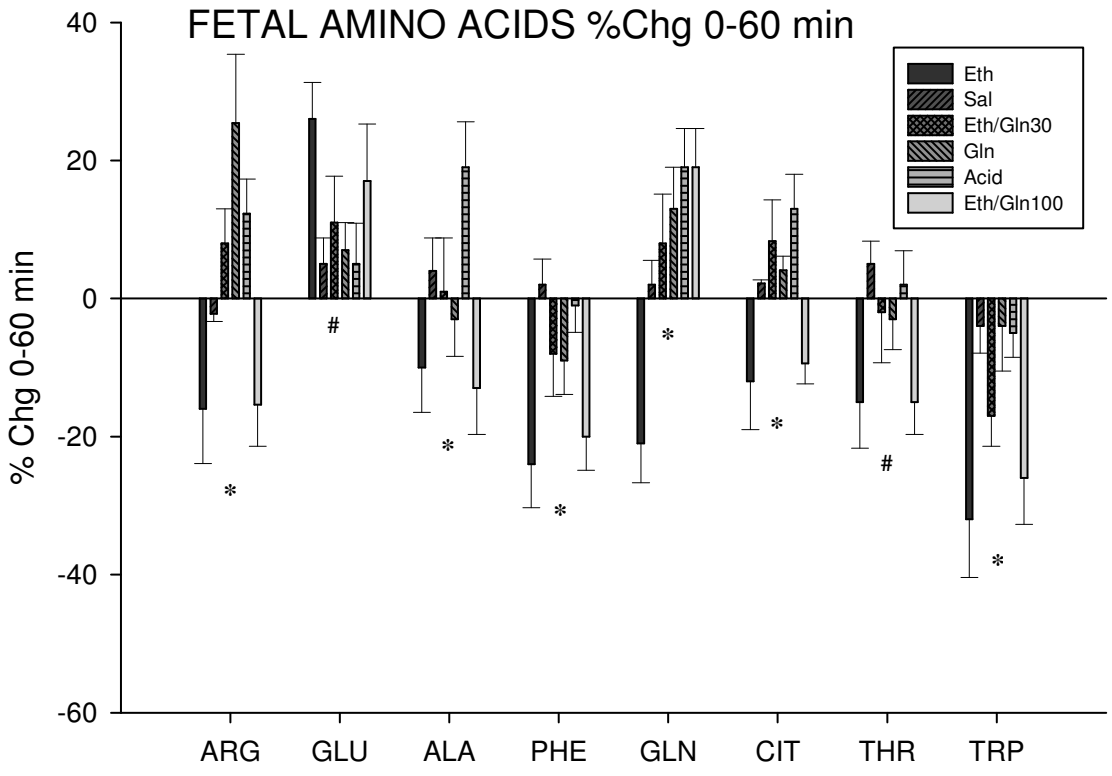

Figure 16: Fetal plasma concentrations of amino acids represented as percent change from 0 to 60 minutes. Significance (*) and trend (#) are indicated on the graph with details on which groups differed in Table 4.

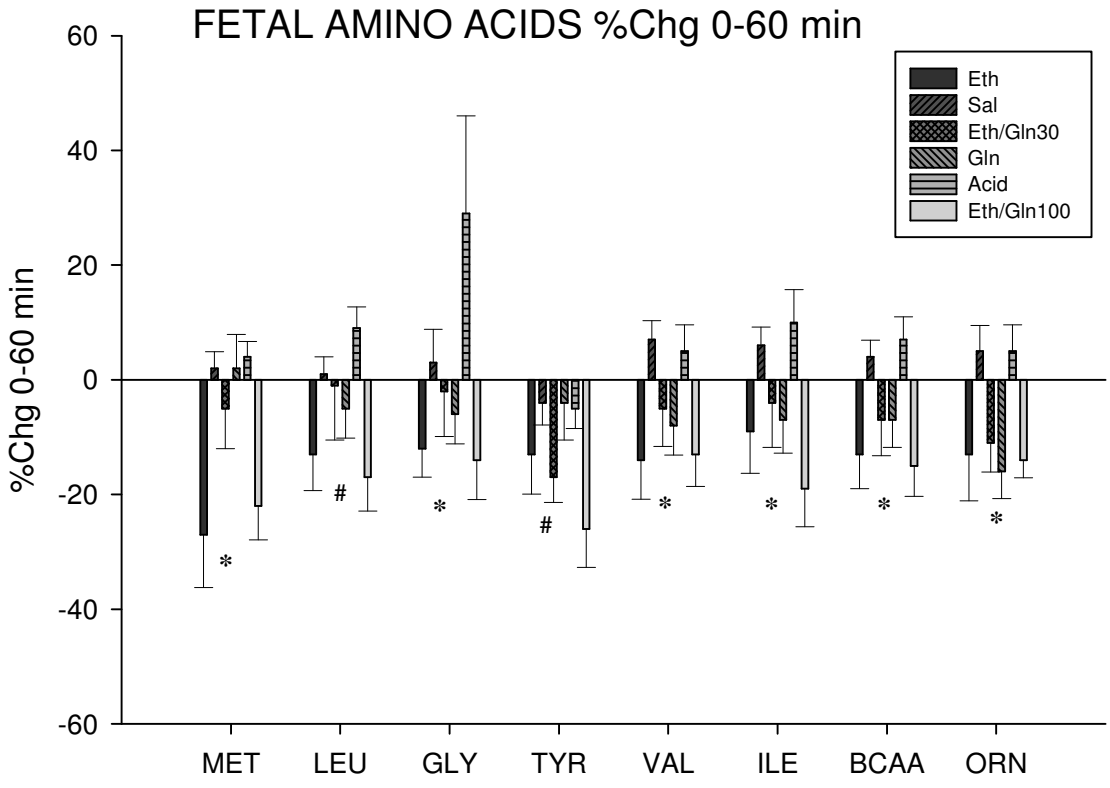

Figure 17: Fetal plasma concentrations of amino acids represented as percent change from 0 to 60 minutes. Significance (*) and trend (#) are indicated on the graph with details on which groups differed in Table 4.

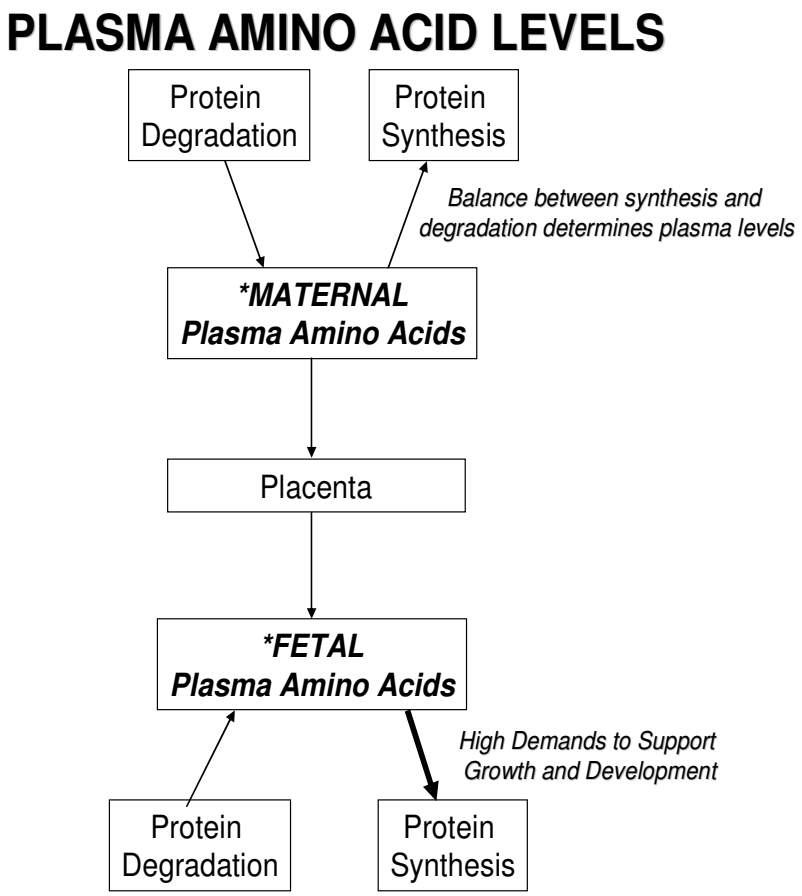

Figure 18: Determinants of plasma amino acid levels

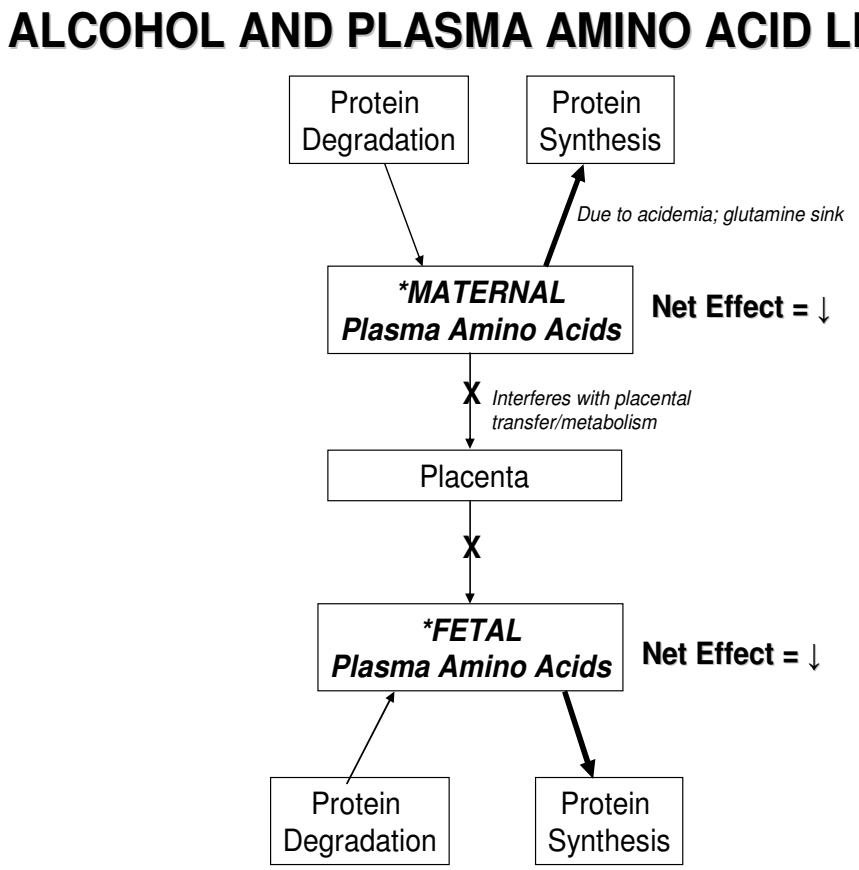

Figure 19: Effects of alcohol on plasma amino acid levels

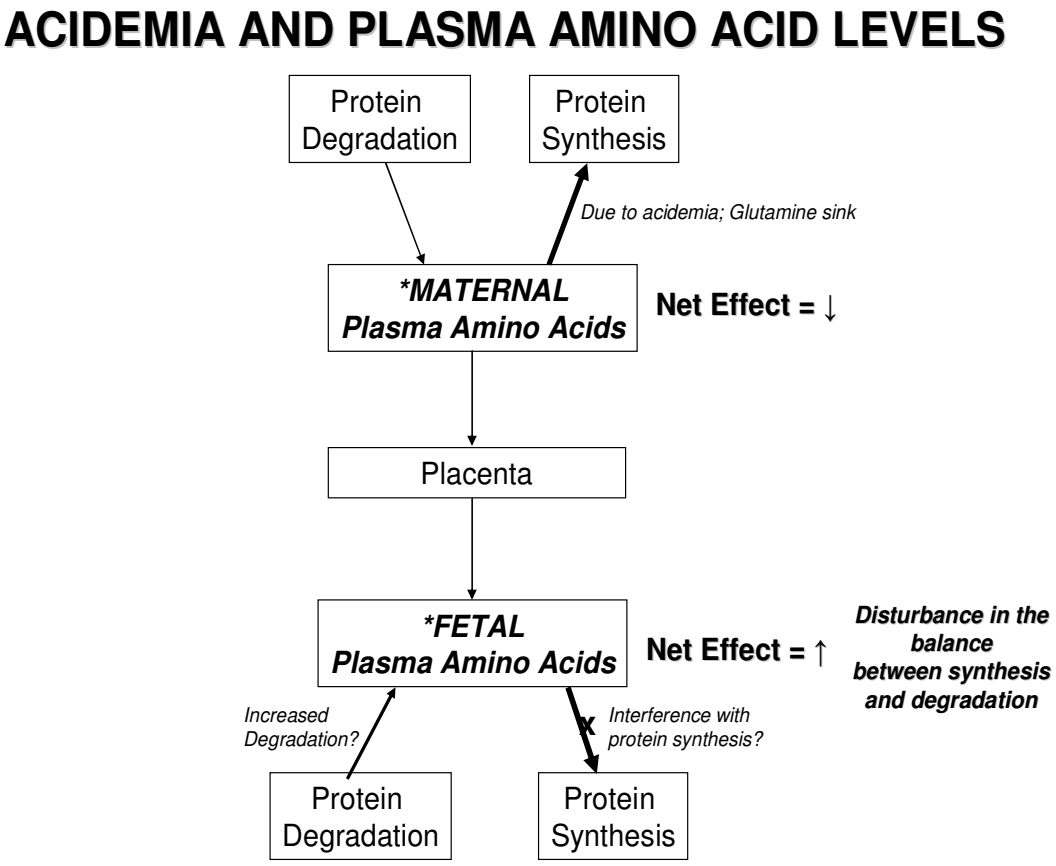

Figure 20: Effects of acidemia on plasma amino acid levels

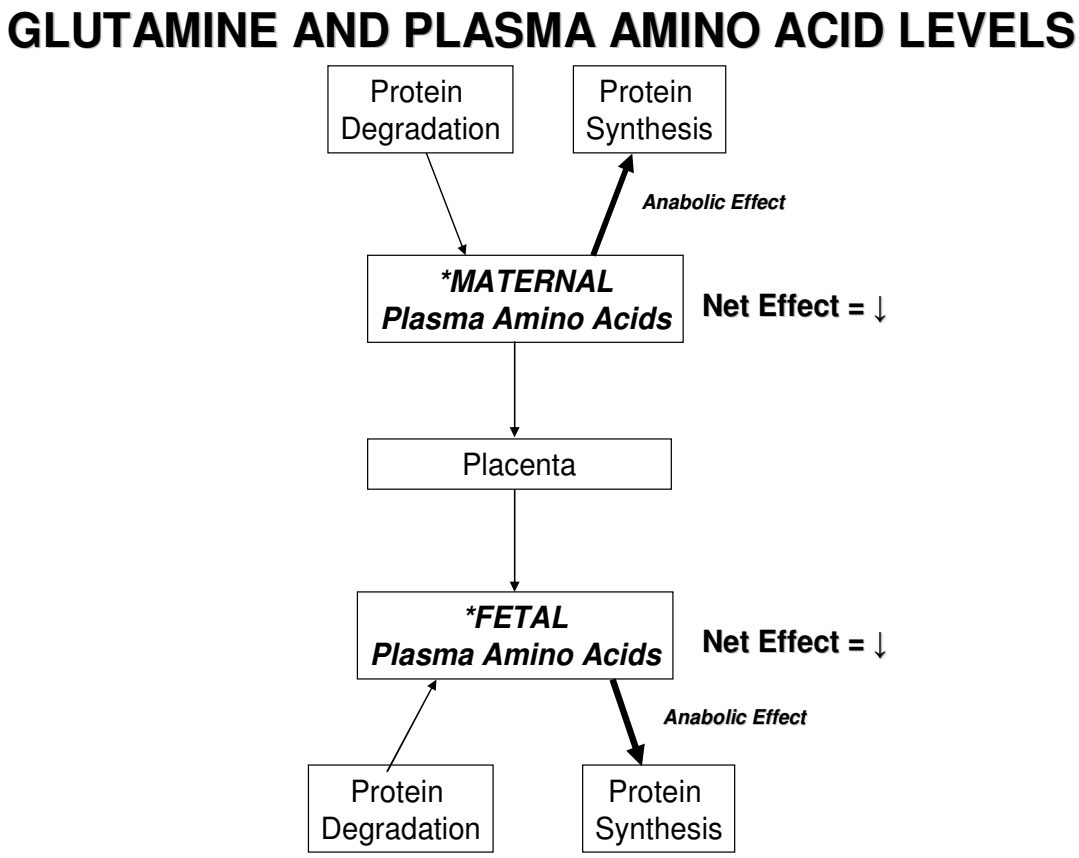

Figure 21: Effects of glutamine on plasma amino acid levels

APPENDIX B

Table 1: Maternal and Fetal pO_2 levels for each group

Group	Mean pO_2 0 min (mmHg)	SEM	Mean pO_2 60 min (mmHg)	SEM
Fetal Saline	12.333	3.019	15.333	3.019
Fetal Alcohol	11.800	2.338	16.000	2.338
Fetal Eth/Gln30	18.833	2.135	15.167	2.135
Fetal Eth/Gln100	15.000	1.849	16.625	1.849
Fetal Acid	11.714	1.976	16.857	1.976
Fetal GLN	18.286	1.976	15.000	3.019
Maternal Saline	89.333	7.772	91.000	7.772
Maternal Alcohol	94.800	6.020	90.000	6.020
Maternal Eth/Gln30	90.000	5.088	83.143	5.088
Maternal Eth/Gln100	88.875	4.759	82.625	4.759
Maternal Acid	89.500	4.759	126.500	4.759
Maternal GLN	92.333	7.772	93.000	7.772

Table 2: Maternal and fetal concentrations of amino acids represented as percent change from 0 to 60 minutes.

Amino Acid	Alcohol	Saline	Eth/Gln 30	Glutamine	Acid	Eth/Gln 100	Signifigance?
ASP *Fetal*	-8 ± 5	-1 ± 3	4 ± 6	2 ± 8	12 ± 8	12 ± 6	No
Maternal	-16 ± 8	6 ± 6	11 ± 8	24 ± 14	-17 ± 5	33 ± 17	Yes
GLU *Fetal*	26 ± 5	5 ± 4	11 ± 7	7 ± 4	5 ± 6	17 ± 8	Trend
Maternal	52 ± 11	-4 ± 2	72 ± 14	1 ± 5	1 ± 6	71 ± 10	Yes
ASN *Fetal*	-21 ± 5	1 ± 4	-6 ± 6	-8 **± 4**	11 ± 3	-17 ± 6	Yes
Maternal	-25 ± 5	-7 ± 5	-10 ± 8	2 ± 7	-13 ± 3	-35 ± 5	Yes
SER *Fetal*	-8 ± 6	0 ± 4	-4 ± 5	-5 ± 5	12 ± 6	-6 ± 6	No
Maternal	-27 ± 7	3 ± 3	-14 ± 9	-9 ± 6	-12 ± 4	-33 ± 5	Yes
GLN *Fetal*	-21 ± 6	2 ± 4	8 ± 7	13 ± 6	19 ± 6	19 ± 6	Yes
Maternal	-25 ± 5	1 ± 1	12 ± 6	30 ± 9	-15 ± 7	86 ± 14	Yes
HIS *Fetal*	-14 ± 7	-1 ± 5	-7 ± 6	-3 ± 6	13 ± 6	-3 ± 12	No
Maternal	-17 ± 4	10 ± 5	6 ± 7	6 ± 4	3 ± 5	-9 ± 7	Yes
GLY *Fetal*	-12 ± 5	3 ± 6	-2 ± 8	-6 ± 5	29 ± 19	-14 ± 7	Yes
Maternal	-22 ± 6	3 ± 7	8 ± 17	5 ± 5	14 ± 9	-22 ± 6	Trend
THR *Fetal*	-15 ± 7	5 ± 3	-2 ± 7	-3 ± 4	2 ± 5	-15 ± 5	Trend
Maternal	-27 ± 6	-1 ± 7	-25 ± 11	-9 ± 5	-4 ± 9	-33 ± 5	Yes
CIT *Fetal*	-12 ± 7	2 ± 0.5	8 ±6	4 ± 2	13 ± 5	-9 ± 3	Yes
Maternal	-26 ± 6	7 ± 6	-22 ± 13	-4 ± 3	-14 ± 5	-10 ± 6	Yes
ARG *Fetal*	-16 ± 8	-2 ± 1	8 ±5	25 ± 10	12 ± 5	-15 ± 6	Yes
Maternal	-20 ± 3	3 ± 2	9 ± 9	10 ± 8	-14 ± 4	-14 ± 5	Yes
ALA *Fetal*	-10 ± 7	4 **± 5**	1 ± 8	-3 ± 5	19 ± 7	-13 ± 7	Yes
Maternal	-25 ± 6	-13 ± 6	-17 ± 10	-5 ± 6	-1 ± 11	-13 ± 6	No
TYR *Fetal*	-13 ± 7	5 ± 4	-8 ± 7	-7 ± 6	11 ± 12	-18 ± 6	Trend
Maternal	-16 ± 6	8 ± 5	-5 ± 9	-13 ± 6	-9 ± 8	-17 ± 5	No
TRP *Fetal*	-32 ± 8	-4 ± 4	-17 ± 4	-4 ± 7	-5 ± 4	-26 ± 7	Yes
Maternal	-19 ± 9	13 ± 5	-2 ± 12	-15 ± 8	-22 ± 8	-20 ± 10	Trend
MET *Fetal*	-27 ± 9	2 ± 3	-5 ± 7	2 ± 6	4 ± 3	-22 ± 6	Yes
Maternal	-17 ± 5	12 ± 2	-16 ± 6	-13 ± 8	-15 ± 5	-14 ± 6	Yes
VAL *Fetal*	-14 ± 7	7 ± 3	-5 ± 7	-8 ± 5	5 ± 5	-13 ± 6	Yes
Maternal	-20 ± 7	8 ± 4	-6 ± 5	1 ± 3	-11 ± 5	-20 ± 6	Yes
PHE *Fetal*	-24 ± 6	2 ± 4	-8 ± 6	-9 ± 5	-1 ± 4	-20 ± 5	Yes
Maternal	-17 ± 4	8 ± 5	-7 ± 6	-2 ± 3	-10 ± 7	-29 ± 6	Yes
ILE *Fetal*	-9 ± 7	6 ± 3	-4 ± 8	-7 ± 6	10 ± 6	-19 ± 7	Yes
Maternal	-17 ± 6	5 ± 4	3 ± 6	-5 ± 3	-7 ± 5	-25 ± 6	Yes

LEU *Fetal*	-13 ± 6	1 ± 3	-1 ± 9	-5 ± 5	9 ± 4	-17 ± 6	Trend
Maternal	-25 ± 7	12 ± 4	-15 ± 10	-7 ± 2	-8 ± 5	-15 ± 6	Yes
ORN *Fetal*	-13 ± 8	5 ± 5	-11 ± 5	-16 ± 5	5 ± 5	-14 ± 3	Yes
Maternal	-1 ± 12	-1 ± 6	9 ± 10	2 ± 5	2 ± 6	-18 ± 6	No
BCAA *Fetal*	-13 ± 6	4 ± 3	-7 ± 6	-7 ± 5	7 ± 4	-15 ± 5	Yes
Maternal	-19 ± 7	12 ± 5	-19 ± 9	-5 ± 2	-11 ± 6	-20 ± 5	Yes

Table 3: Maternal amino acid concentrations with significance among groups

	ALCOHOL (a)	SALINE (b)	ALC/GLN30 (c)	GLN (d)	ACID (e)	ALC/GLN100 (f)
ARG	b,d	a	d	a,c,e,f	d	d
GLU	b,d,e	a,c,f	b,d,e	a,c,f	a,c,f	b,d,e
PHE	b	a,e,f	f	f	b,f	b,c,d,e
GLN	b,c,d,f	a,d,f	a,e,f	a,b,e,f	c,d,f	a,b,c,d,e
CIT	b,d	a,c	b	a		
THR	b,e	a,c,f	b	f	a,f	b,d,e
LEU	b,d	a,c,f	b	a		b
VAL	b,d	a,e,f		a,f	b	b,d
ILE	b,c	a,f	a,f	f	f	b,c,d,e
BCAA	b	a,c,e,f	b		b	b
SER	b	a,f		f	f	b,c,d,e
HIS	b,c,d,e	a,f	a,f	a,f	e	b,c,d
ASN	b,d	a,e,f	f	a,f	b,f	b,c,d,e
ASP	c,d,f		e	a,e	c,d,f	a,e
MET	b	a,c,d,e,f	b	b	b	b

a = statistically different compared to alcohol group
b = statistically different compared to saline group
c = statistically different compared to alcohol + glutamine 30 mg/kg group
d = statistically different compared to glutamine group
e = statistically different compared to acidemia group
f = statistically different compared to alcohol + glutamine 100 mg/kg group

Table 4: Fetal amino acid concentrations with significance among groups

	ALCOHOL (a)	SALINE (b)	ALC/GLN30 (c)	GLN (d)	ACID (e)	ALC/GLN100 (f)
ARG	c,d,e	d	a,f	a,b,f	a,f	c,d,e
ALA	e				a,f	d
PHE	b,c,d,e	a,f	a	a	a,f	a,e
GLN	b,c,d,e,f	a,e,f	a	a	a,b	a,b
CIT	c,e		a,f		a,f	c,e
TRP	b,d,e	a,f		a,f	a,f	b,d,e
MET	b,c,d,e	a,f	a	a,f	a,f	b,d,e
GLY	e		e	e	a,c,d,e,f	e
VAL	b,e	a,f			a,f	b,e
ILE	b,e	a,f			a,f	b,e
BCAA	b,e	a,f			a,f	b,e
ORN	b,e	a,b,c,f	b,e	b,e	a,c,d,f	b,e
ASP	b,c,e,	a,f	a,e	e	a,c,d,f	b,e

a = statistically different compared to alcohol group
b = statistically different compared to saline group
c = statistically different compared to alcohol + glutamine 30 mg/kg group
d = statistically different compared to glutamine group
e = statistically different compared to acidemia group
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